IMPROVING INDUSTRIAL PERFOMANCE OF *Chlamydomonas reinhardtii* THROUGH GENETIC ENGINEERING: A FOCUS ON STRESS TOLERANCE AND IRON TRANSPORT.

by

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ABSTRACT

Algal cultures exhibit the potential as robust systems for pharmaceutical production, as sustainable sources of nutrition both for humans and livestock, and as renewable biomass feedstock for biofuel production. Algal biomass grows quickly, requires minimal nutrient supplementation and sunlight, and has the ability to sequester environmental CO_2. Although these properties seem attractive, algal industrial cultivation is still far from optimal; leading to non-ideal culture conditions which induce cellular stress that cause a loss in productivity. Furthermore, bioprocessing of algal biomass for biofuel production is still in its infancy and encounters many obstacles and bottlenecks after mass cultivation, mainly cell separation from culture.

In this study we aim to address these industrial issues by employing the power of genetic and metabolic engineering. Specifically, we employ a two-fold plan to increase the stress tolerance and intracellular iron concentration of model organism, *Chlamydomonas reinhardtii*, by overexpressing mammalian anti-apoptotic BCL-XL protein and the native membrane coupled iron transporter IRT2. In order to achieve our goal a gene construct driven the native hsp70/rbcS2 tandem promoter was constructed containing the appropriate gene of interest and carrying a Hygromycin B

resistance marker for downstream selection. After transformation, resulting colonies were screened via colony PCR; positive colonies were subcultured and exposed to a variety apoptosis inducing agents tailored to mimic stresses involved with industrial cultivation; mainly photooxidative damage, exposure to reactive oxygen species, osmotic pressure change, and intracellular damage caused by high irradiance. While the transgenic cell line experienced a reduced growth rate, it reached a higher cell density and featured higher cell longevity in comparison to the wild type after prolonged culture. Furthermore, in all stress related experiments the transgenic cell line outperformed the wild type, often with stark phenotypic changes. Moreover, RT-PCR analysis confirmed RNA level expression of this peptide allowing us to conclude that the transgenic cell line was producing a functional form of this recombinant anti-apoptotic protein leading to more robust stress tolerance.

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Today's growing population and humanity's demand of energy are increasing, along with the increased consumption and subsequent scarcity of non-renewable resources. These perishable resources include fossil fuels such as petroleum, natural gas, and products that are derived via refinery. BP estimates that our current Energy consumption rates will cause an energy crisis in America within 55 years [1]. Certainly, there are other more efficient methods of energy production including nuclear, solar, hydro-electric, wind powered, and coal powered; however, some resources are deemed "too risky", while others are very damaging to the environment. Furthermore, while "greener" options are very attractive there is simply not enough infrastructure currently in place to support American society or our consumption habits.

In order to preserve our lifestyle, ensure our survival, and restore our environment; we have looked at various other forms of renewable resources, an attractive option being a shift to biofuels. Biofuels are attractive because they provide a relatively cheap renewable source of biomass that can be used as a precursor or producer of combustible products. In the past, a few sources of biomass have been recruited such as wood, corn, soybeans,

sugarcane, and other high carbohydrate and lipid containing plants. Biofuels are usually derived from plants which use photosynthetic processes to convert nutrients in the soil into biomass using sunlight and CO_2 provided by the air. Additionally, other plant components such as lipids, combustible or fermentable hydrocarbons, and other high value biochemicals are valuable byproducts of plant biomass. Thus biofuel production is inherently less damaging to the environment than fossil fuel derived products. Unfortunately, limitations such as long production and harvesting times, biological constraints, and downstream processing bottlenecks plague the industry and drive cost of production up.

In recent years however, funding into alternative fuel research has provided useful solutions that have decreased the cost of biofuel production. Better cultivation methods and a deeper understanding of an organism's metabolism allows us to optimize nutrient feed conditions in order to induce specific product generation. Careful analysis and strain selection allows us to identify the optimal organism for the production of a particular product. Taking the advantage of the biological nature of our source we can genetically modify our organisms to produce what we desire. Another lucrative property of biofuel production is that it is essentially carbon

neutral; meaning it leaves little to no carbon emission which greatly benefits our environment. Lastly, in recent years giant leaps have been made in order to make biofuel production Carbon negative, which would benefit us as a society by greatly reducing our carbon footprint.

1.1 Biofuels: A Timeline

Biofuels are by no means a novel concept; ancient human beings used solid forms of plants such as dried leaves and wood as fuel to feed their early heating systems. Much later, extraction processes were discovered which allowed us produce vegetable and plant oils which were used as higher energy density liquid fuels, ointments, or other forms of raw goods. If fuels derived from plant-based matter have been known to humans essentially since the beginning of time, then why is our energy economy not entirely based on biofuels? The answer to that question lies in the colossal demand of energy that our society requires; advances in civilization, the advent of electricity, and our boom in population caused an exponential increase in our energy consumption. Furthermore, this exponential demand for fuel could not be met by merely burning wood and or plant matter; therefore, we turned to using fossil fuels. We became dependent on fossil fuels during the industrial revolution because these fuels provide a much higher energy

output per mass than biofuels could. Thus we centralized our energy economy around fossil fuel production and refinery, never really thinking about the harrowing consequences this would have on our environment.

Fortunately, in recent years, advances in chemical engineering, biofuel processing, and industrial process development allow us to now chemically, physically, and or mechanically treat raw plant materials in order to produce higher energy density products. Some of these products include biologically produced diesel, alcohols, hydrogen, and crude oil amongst other high energy density liquid and gaseous hydrocarbons. This again opens the opportunity to decrease our dependence on fossil fuels and turn to biofuels as an alternative cleaner burning option.

First Generation Biofuels:

We have discussed how biomass from plants was used by ancient civilizations for heating and cooking purposes. Our aim is to elucidate how bio-processing allowed us to switch from using renewable biomass as a fuel to using it as a direct precursor for higher energy density compounds. We begin our discussion by defining what is commonly referred to as a first generation biofuel in industry. First generation biofuels are usually derived

from edible food sources such as crops like corn, sugar and vegetables, animal fats and plant oils [2]. The plant based starting materials are used in various processes including fermentation, enzymatic degradation, anaerobic digestion, and extraction and transesterification processes which ultimately produce higher value biofuels [3].

The first step in these biofuel production processes largely consist of breaking down raw feedstock into their simple sugars [4]. These simple sugars are in turn fed into a fermentation and or anaerobic digestion process in which various organisms consume the feedstock and produce either alcohols such as methanol, ethanol and butanol and or gasses such as methane [5]. These gasses produced can be collected and separated downstream from contaminating components, purified and subsequently used as a fuel source. Another common process involves extracting essential oils from the feedstock through thermal, chemical, or mechanical treatment and then feeding the fatty oils through a transesterification process which produces biodiesel and other high energy density hydrocarbons [6]. While these processes produce viable energetic alternatives to fossil fuels, cost of production and raw material availability plague the industry with obstacles.

Second Generation Biofuels:

Second Generation biofuels are a lot like first generation biofuels in that they are also derived from plants which use photosynthesis in order to produce biomass. Second generation biofuels differ from first generation counterparts because instead of using edible feedstock, fibrous and lignocellulosic crops are used as raw materials. Lignocellulosic crops are more abundantly available than feedstock crops and contain many of the same carbohydrates [7]. The drawback on these kinds of plants is that their biomass contains aromatic compounds such as lignin and terpenes which are inherently harder to break down into simple sugars than the carbohydrate polymers found in feedstock crops [8]. Fortunately, we have multiple processes that allow us to overcome the lignin obstacle and use these non-essential crops as a source of biofuels.

Obviously, in order to produce biofuels efficiently from lignocellulosic plants we must be able to break tough lignin containing tissues into smaller more readily usable carbohydrates. Lignin is chemically composed of highly cross linked phenolic polymers which are recruited to hard tissues such as bark or cell walls of plants [9]. Fortunately, we have an array of different chemical, mechanical, and biochemical methods of

breaking down lignin. One major method of breaking down lignin is through thermochemical processes, mainly Gasification and Pyrolysis. Gasification is a process which uses high temperature oxygen or steam in order strip hydrogen from biomass which can in turn be used as fuel [10]. Pyrolysis is another thermochemical process which produces oils by heating biomass in an anaerobic environment [11]. The other widely used process for the breakdown of lignin is achieved by enzymatic degradation [12]. This biochemical process uses enzymes which use lignin as a substrate and leave simple sugars as a product; the simple sugars are then converted to fuel via fermentation [13].

Third Generation Biofuels:

Switching raw material sources for second generation biofuels mitigates the problem of using food as precursors to fuel and has had an extensive effect on biofuel production. However, cultivating lignocellulosic plants presents a challenge which has shifted the focus on biofuel precursors. Specifically, lignin containing plants while very abundant have long life cycles and many can only be planted and harvested during particular points of the year [14]. Furthermore, these kinds of organisms serve our environment by removing CO_2 from the air and replacing it with oxygen. If

we were to only rely on these organisms for biofuel production eventually the benefit of producing fuels with a small carbon footprint would be outweighed by the amount and rate of deforestation [15], essentially a catch 22. In order to counteract this issue the industry shifted to algal biomass as a precursor to fuel.

But why switch to algae? The answer is simple, the algae used for biofuels are unicellular organisms that according to the U.S DOE can yield up to 30 times more energy per acre than land crops can. Algae are also very attractive because they are relatively easy to cultivate and have fast growth rates and can be grown year round [16]. The benefit of using algae boils down to cheap and readily available raw materials for growth, these include: water, sunlight, a phosphate source, a nitrogen source and trace metals [17]. Like first and second generation biofuel precursors they use photosynthesis to produce biomass quickly, which in turn can be turned into fuel. An additional benefit of algae is that they are relatively simple organisms which can be genetically modified in an attempt to seek better growth or a metabolic shift towards a particular goal [18].

Current Advances in the Biofuel Industry:

Currently, there is an extensive amount of research being done on algae in order to make it a more viable and higher yielding source for biofuels. In addition, the biofuel industry is beginning to look into the idea of making "carbon negative" processes; meaning that they are actively trying to capture CO_2 and other greenhouse gasses from the air and from industrial processes by using either microorganisms or by geo-sequestration [19]. The aim of these processes is twofold: to reduce our carbon footprint and to produce fuel economically.

1.2 Current Limitations of Biofuel Production

All alternative energy sources face an uphill battle; especially when considering that most of the worlds' infrastructure is powered by fossil fuels [20]. In recent years however, we have seen the production of hybrid and electric cars, and an effort from large motor companies to produce smaller more efficient engines. This points to a bright future for much needed biofuel research.

The biofuel production industry is relatively new and although biofuels research is advancing rapidly, the industry is still plagued with

many obstacles and bottlenecks. These bottlenecks are significant as they raise the cost of fuel production and generally retard the process as a whole [21]. Many challenges such as the 'food vs. fuel' argument and the energy per acre issue that burdened first and second generation biofuels have been mitigated by algal cultivation. Even so, algal biomass while a viable alternative to fossil fuels is still not a more profitable alternative [22].

Algal culture is a cheap, environmentally friendly, and quick method to obtain lipid and carbohydrate rich biomass that can be readily processed into biofuels. Algae can grow either suspended in photo-bioreactors (PBR's), or in bio-films under appropriate conditions [23, 24]. Culture and maintenance of PBR's is a minimal cost; in fact, sunlight could be used as a light source and often is in raceway pond culture [25, 26]. The biggest bottleneck in algal processing is not at the culture stage, but rather at the harvesting stage [27]. Algae, in order processed, must first be 'dewatered' or separated from suspension. There are a few ways to do this; the least expensive being gravity sedimentation. Unfortunately, gravity sedimentation is very slow and not industrially viable [29]. Thus centrifugation is the quickest most common way of dewatering algae; however, when dealing with large quantities of material (thousands of liters) this process becomes

very cumbersome and extremely expensive [29]. Other alternatives such as flocculation is also applied but again are either very costly, have low separation efficiency, or include the addition of chemical or magnetic agents which require downstream separation [30].

Biofuel production costs will reduce to a profitable level once more efficient algal separation processes are developed however; one cannot disregard the inherent nature of working with biologics. Cell culture is very delicate and requires extreme care and diligence; any tiny mistake can contaminate the culture and cause a failed batch [31]. Furthermore, in the biofuel production industry batch to batch variation is the norm; unlike chemical reactions which can be modeled and predicted, an organisms' development can only be expected to follow a common behavior. Additionally, microorganisms in culture are very sensitive to sudden changes in environment; sudden stresses can change an organism's behavior unpredictably [32]. Lastly, after rounds and rounds of cell division mutations tend to accumulate in the strains used for production which can cause unwanted consequences and loss of productivity [33]. This manuscript aims to address the current industrial shortcomings in algal stress

tolerance and separation techniques through a genetic and metabolic engineering approach.

Chapter 2: The role of Algae in Biotechnology

With the advent of third generation biofuels algal biomass became a very attractive resource for various reasons, mainly because it is cheap to produce and grows quickly. Algae are an abundant class of Eukaryotic photosynthetic organisms that grow in diverse aqueous environments. Algae are further subcategorized into unicellular and multicellular organisms; in the interest of biofuels research we will focus on unicellular algae.

2.1 General Properties of Microalgae

Microalgal biomass is composed of significantly more lipid and carbohydrate content than multicellular algae [34, 35]. Furthermore, microalgae grows much faster than multicellular algae, thus it presents a potential as a source of renewable biomass [36]. Like land plants, algae use sunlight and atmospheric carbon dioxide in order to grow, contrastingly microalgae do not exhibit roots, stems, leaves, or complex vascular structure [37]. They are a very abundant class of organisms that grow in suspension within an aqueous environment. There are an estimated 200,000 subspecies that can be found living in both freshwater and marine systems [38]. Microalgae exhibit a fast growth rate with most known species having a

doubling time of less than 24 hours [39]; this allows a faster biomass turnover rate which is optimal for biofuel production.

Algae accumulate lipids as triacylglycerides as oily lipid bodies [40] which can be converted into biodiesel through pyrolysis and can be grown in non-sterile and waste water sources [41, 42]. Additionally many algae, depending on their environment, can change their biomass composition and adjust their metabolism to grow under environmental stress, making them robust industrial workhorses [43]. It is important to note that this adaptive quality is very attractive to industry; mainly because it presents opportunities where culture conditions can be adjusted in order to accumulate or generate a desired product. An example of this phenomenon is the production of hydrogen by Chlamydomonas reinhardtii under sulfur deprivation [44]. Algae are mostly photosynthetic and grow photoautotrophically however; many algae can utilize organic carbon sources like glucose and acetate for heterotrophic growth [45]. In general, algae require water, light, carbon dioxide, nitrogen, iron, and phosphorous to grow [46]. In addition to being an optimal source of biomass for biofuel production algae can be exploited for a variety of other uses including pollution control, industrial carbon sequestration, fertilizer, feedstock for fish, bioremediation and water

treatment [47, 48, 49, 50, 51, 52, 53]. These organisms can also form symbiotic relationships with fungi or other cyanobacteria, this behavior can be exploited in an effort to create robust co-culture systems [54, 55, 56, 57]. Lastly, many species of microalgae can be used directly for the production of high value biochemicals including antioxidants, carotenoids, and biopolymers; clearly there is vast untapped potential in microalgal culture [58, 59].

2.2 Industrial Algal Cultivation Methods

We have discussed algal biodiversity, biochemical composition, biological properties and requirements for growth in the wild. However, in order to make algal cultivation a feasible source of biomass we must be able to culture it industrially. During algal culture it is extremely important to monitor and control growth. This is done industrially by employing the use of either aqueous culture, or in some cases growth as a biofilm on a solid surface [60]. It should be noted that most industrial algal cultivation is done through photoautotrophic aqueous culture because it offers the highest biomass productivity rate.

Open Raceway Ponds:

The most common process for mass algal cultivation is in an open raceway pond. In this system a raceway made of concrete is built which holds column of water about 1 foot deep [61]. This system mimics natural algal growth conditions and takes full advantage of sunlight as it is open to the atmosphere. A paddle wheel spins and causes the media to flow circulating nutrients and mixing the culture improving mass transfer and ultimately growth conditions [62]. These systems can be run in either a batch, fed batch, or continuous mode and allow large amount of biomass to be produced relatively quickly [62]. Fresh feed is added initially and it flows along the track being consumed and producing biomass along the way and the biomass is harvested at the end of the circuit. Some of their many attractive features include that they are cheap to build and maintain, they can be optimized for surface area, and they can use wastewater or at times even seawater as a growth substrate thus reducing costs even further [63].

While open raceway ponds are an attractive and simple option for mass algal cultivation they have numerous shortcomings. The first, most obvious concern is contamination. If the system is being fed with a carbon source such as glucose many times faster growing bacteria will contaminate

your culture and outgrow algae, effectively ruining a batch. Another issue also associated with the environment water loss in the system due to evaporation [64]. Since this is an open air system mass transfer between the air and the water is very limited, therefore limiting cell growth and decreasing biomass productivity [64]. Lastly, due to sub optimal growth conditions these cultures tend to be dilute at the time of harvest this increasing downstream separation costs.

Schematic Representation of an Open Raceway Pond

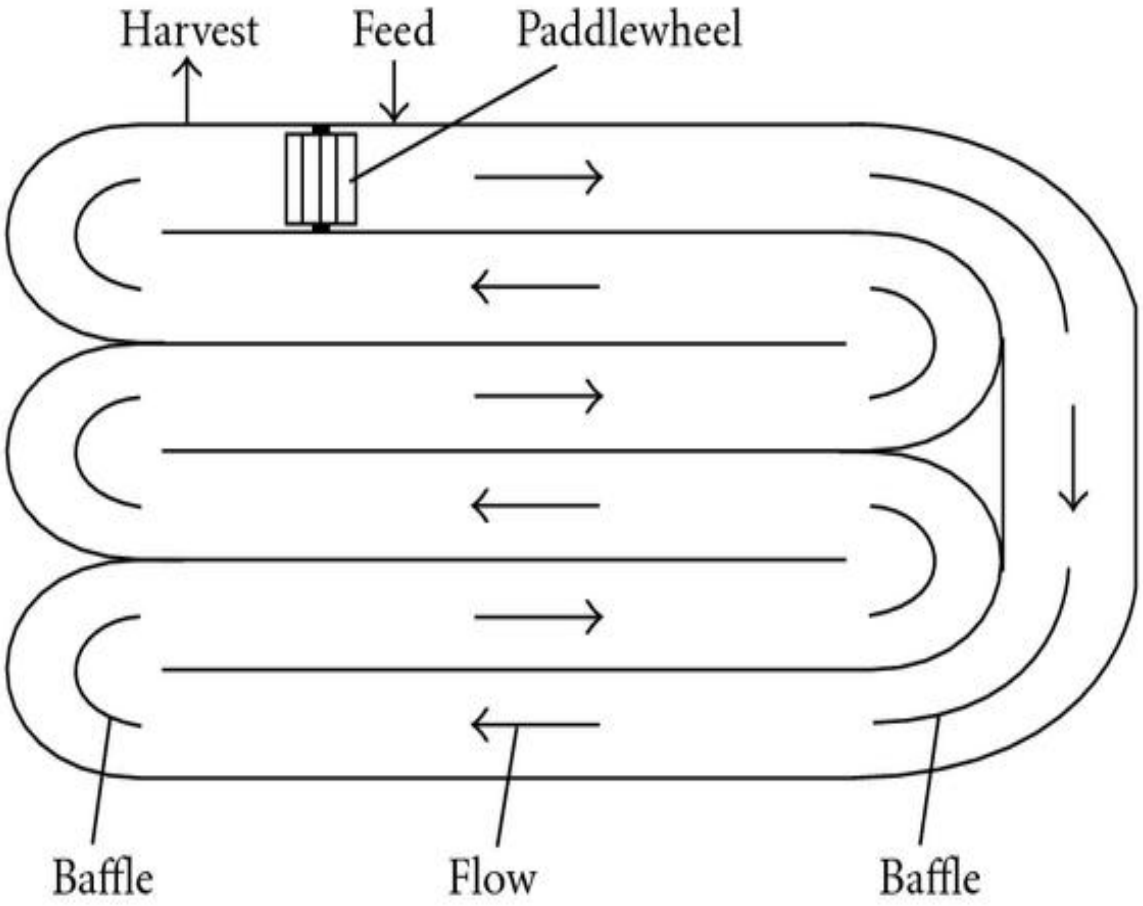

Figure 1. Open raceway ponds are the most common processes for the mass cultivation of microalgae. They offer a simple, cheap, and effective cultivation conditions by taking advantage of land surface area and since they are built in the open; sunlight. Flow in these systems is driven by a paddle wheel and the raceway ensures that cells flow from starting point to harvest point. Drawbacks with these systems include inefficient transfer of CO_2 and sunlight, contamination, and frequent stress causing disturbances [142].

Photobioreactors (PBR's):

While open raceway ponds provide biomass in a simple and effective manner we can increase biomass productivity by using photobioreactors (PBR's). PBR's are sealed chambers made of transparent materials such as glass or clear polymers in which biomass is produced. PBR's solve environmental factors such as contamination and evaporation which is attractive because it mitigates batch to batch variation [65]. These systems can be placed outside to take advantage of natural sunlight or can be illuminated artificially. Algal growth in PBR's is inherently easier to control than in open ponds because optimal gas mass transfer can be achieved allowing cells to sustain optimal growth rates [66]. Additionally, flow in these reactors is achieved via fluid or air pump increasing the liquids Reynolds number and establishing a turbulent flow profile allowing optimal nutrient availability for cells [23].

The most commonly used PBR design for biomass production is a series of tubes made of clear glass optimized for maximum sunlight penetration. This careful design allows algae to have the most amount of sunlight available allowing them to achieve maximum growth potential [67]. These reactors save space and can be run on batch, fed-batch, or continuous

mode [67]. These reactors also decrease downstream separation costs because they concentrate the media with up to 30 times more biomass. While these reactors offer effective solutions to problems encountered with open raceway ponds and offer higher biomass productivity they do have some shortcomings. One of the shortcomings comes as a product of photosynthesis, Oxygen [68]. In an open raceway pond oxygen accumulation is not a problem as the system is open to the environment. However, oxygen accumulation in PBR's is an implicit issue that will inhibit photosynthesis killing algae [69]. Additionally a high concentration of oxygen in the media coupled with direct sunlight can catalyze the formation of reactive oxygen species which also kill algae [70]. Therefore degassing systems must be put in place to deal with this issue, which adds to the capital cost. Additionally, the upkeep and scale up of these systems is more difficult and expensive along with the necessity for these systems to be highly customizable [71]. Lastly, as cell concentration increases in these reactors biomass productivity decreases as essential nutrient, light, and CO_2 quotas are not met due to poor mass transfer [66].

Representative Diagram of a Tubular Photobioreactor

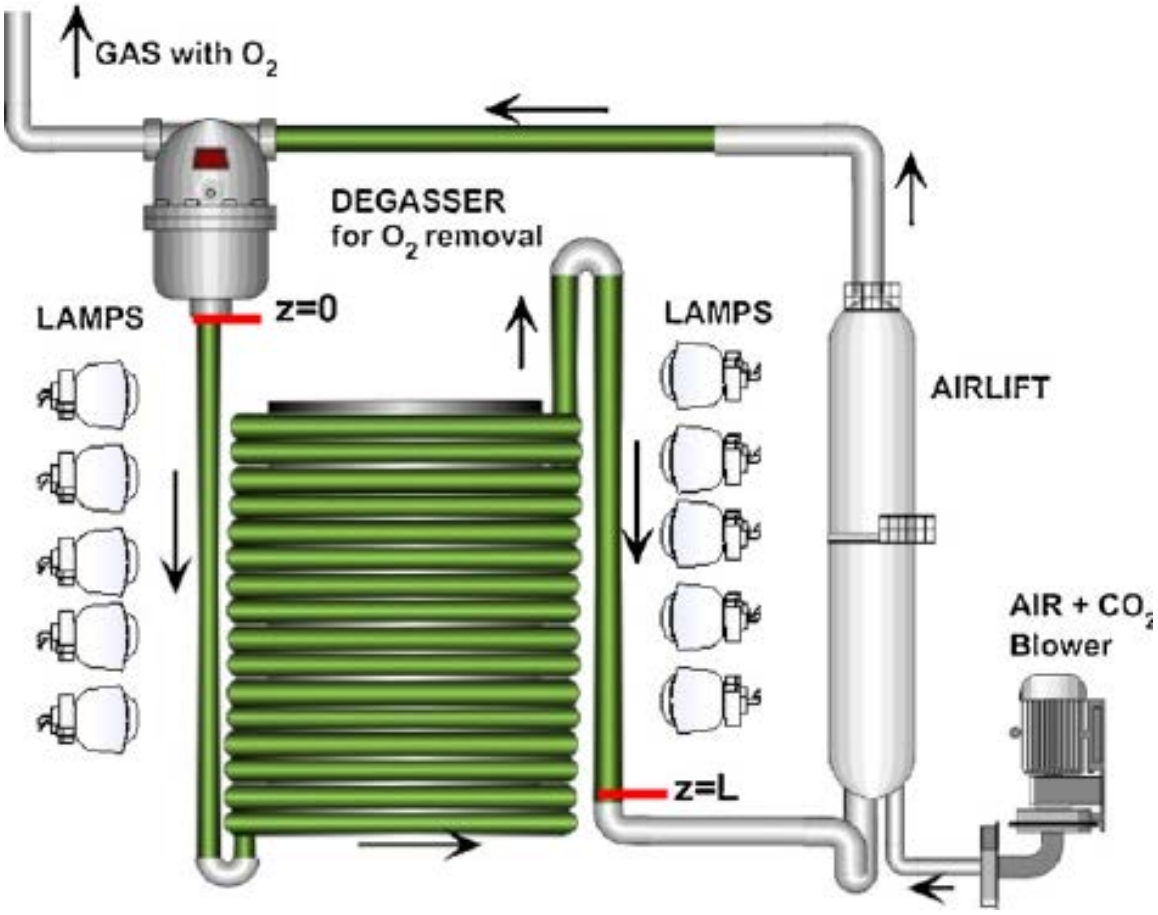

Figure 2. Diagram of a typical tubular photobioreactor; these systems yield higher biomass productivity than open raceway ponds but are more expensive to build and maintain. These reactors can be built outdoors in order to maximize sunlight usage or indoors with synthetic illumination. These systems usually use pumps that pressurize air to drive flow through the channels. CO_2 from the air is utilized and at the end of the channel oxygen produced by photosynthesis is expelled in an effort to limit the formation of ROS catalyzed by sunlight [143].

Biofilm Growth on Solid Surfaces:

As mentioned before algae can form biofilms in certain situations, the most common being when grown on solid nutrient agar containing media [72]. Once inoculated colonies will form on the solid media and grow in an outwards fashion. Another common industrial way of spurring biofilm growth is by employing the use of an organic matrix as a scaffold for biofilm formation [73]. On this scaffold algal biofilm forming capacity is exploited aiming at finding symbiotic relationships to form co-cultures. A study done in 2016 showed that Mucor *sp.* and *Chlorella* sp. would increase total biomass when grown in co-culture as opposed to either in monoculture [74]. Aside from the fact that many times these symbiotic relationships can be beneficial for the growth of either organism, many times certain biological behaviors can only be achieved through co-culture [75]. Additionally, when algae are grown in biofilms harvesting is inherently easier since separation from water is not required.

Algal Growth in Biofilms

Figure 3: Algae can be grown in a biofilm attached to a matrix; this figure shows revolving film reactor. Algae are attached to a revolving film where they are gradually exposed to nutrients. This system allows the maximum surface area for sunlight exposure and allows maximal CO_2 exposure. Additionally, since not grown in suspension the dewatering process is less extensive. (Images courtesy of *Martin Gross, Iowa State University|https://www.aiche.org/chenected/2013/11/rotating-algal-biofilm-reactor-on-location*)

2.3 Improving Algal Industrial Output through Genetic Engineering

Presently algae are a staple of the biotech industry and serve as a reliable and fast organism for producing biomass. Algae, being biological organisms, present the opportunity for genetic engineering. We can employ algae as biological 'micro-factories' in an effort to enhance their productivity, growth, or even biomass composition [76]. By studying their metabolic pathways we can edit their genome and tailor it to our specific needs. Much research has been done in developing tools for the successful incorporation of transgenes including sequencing of various species nuclear, mitochondrial, and chloroplast genomes [18]. Currently, we have transformed more than 30 strains of microalgae; however, only a few algal strains have been identified that can produce stable transformants [77]. Even so, in recent years algal genetic research has been a topic of discussion and new methods of gene incorporation are being developed.

Microalgae are a viable candidate for genetic engineering because they are fast growing, unicellular organisms with relatively simple metabolisms. This feature allows us to more easily understand the underlying mechanisms that drive their metabolism and ultimately tailor it for the production of valuable bio-molecules or biomass [76].

Metabolic Pathways of Interest for Algal Genetic Engineering

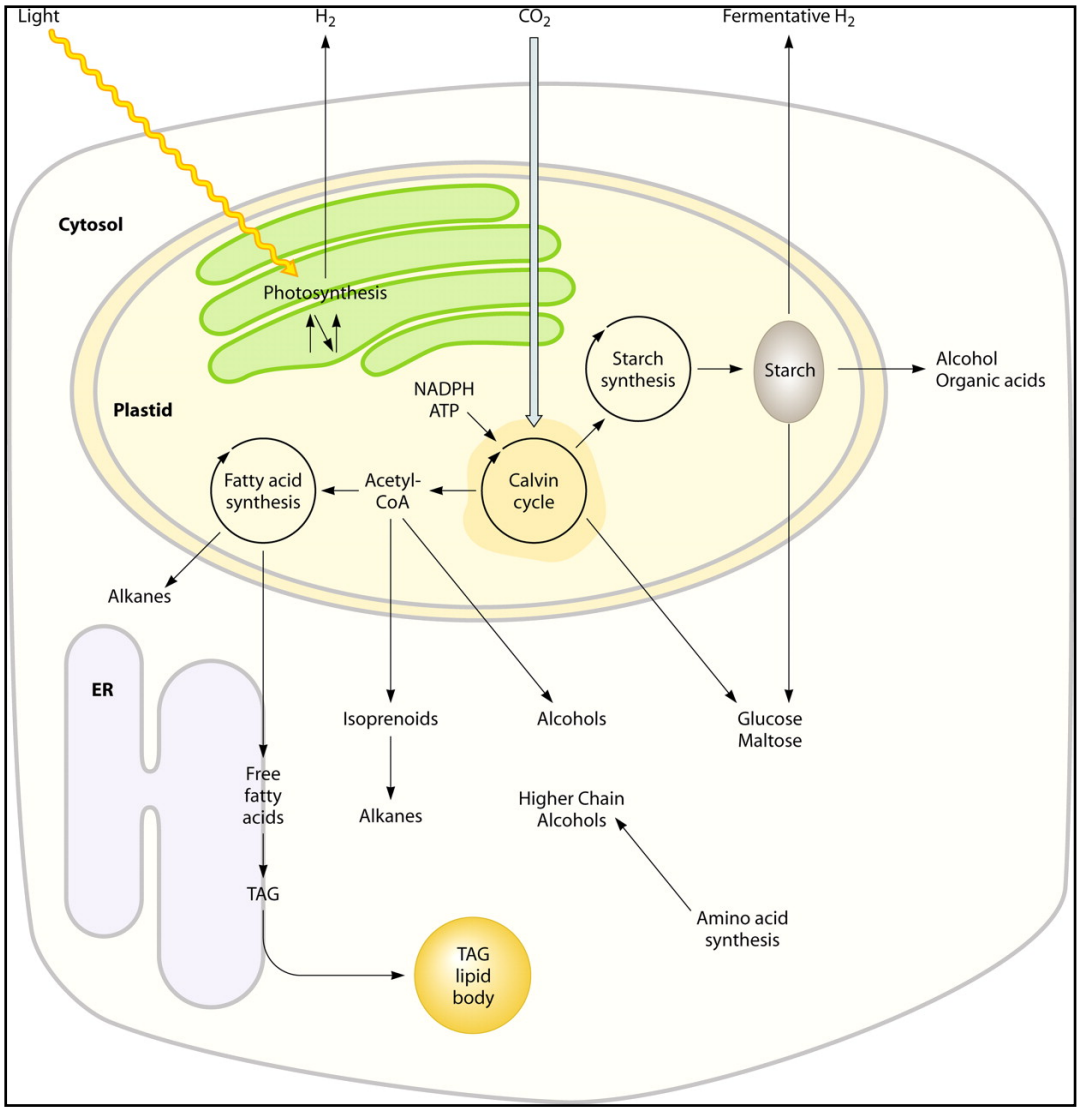

Figure 4. Microalgae are unicellular eukaryotes that exhibit fast growth rates. This presents an opportunity for researchers to exploit these pathways in an effort to increase the production of a valuable product. For example, the lipid metabolism can be leveraged for biofuel production and the photosynthetic apparatus for Hydrogen production [144].

In the past work has been done that has successfully yielded higher lipid accumulation, higher starch accumulation, knockout of complete metabolic pathways and other gene editing techniques[78, 79, 80]. Some areas of current research include the use of metabolic engineering to enhance triacylglycerol synthesis and accumulation, enhance the yield and production rate of hydrogen, and to improve the carbon capture rate of algae [79, 80, 81].

Clearly, the biofuel industry is headed towards a cheap method of biomass cultivation with bioengineered physiological traits however; the biofuel industry does not exclusively benefit from bioengineered algae. As mentioned before, research is being done to improve carbon capture rates in an effort to use algae as agents for bioremediation. Work is also being done in order to increase the synthesis of astaxanthin and other carotenoids for use in the food industry [82]. Thus, genetic engineering proves to be a promising technique for the enhancement many facets of algal industrial output.

2.4 A Focus on *Chlamydomonas reinhardtii*

As mentioned before genetic engineering with microalgae is a relatively new concept and while many approaches have been attempted the

subject is still in its infancy. The reason that algal genetic transformation is difficult is because transgenes are integrated into the nuclear genome at random [83]. This random integration causes issues with efficient expression, furthermore; high rates of foreign gene silencing also causes difficulty establishing stable transformants [84]. Although algal transformation is difficult a few strains have risen as model organisms for genetic studies, mainly *Chlamydomonas reinhardtii.*

The algal strain *Chlamydomonas reinhardtii* has been the workhorse of algal genetic research because its' genomes have been sequenced and annotated, and stable nuclear transformation is possible. This strain of microalgae is unicellular flagellate found in fresh and seawater and has become a model organism not only for genetic but also photosynthetic, phycological, flagellar motility, and general biological studies [85]. Additionally, this strain is both an autotroph and a heterotroph; thus it can be grown mixotrophically [86]. Most methods for algal genetic transformation were designed for use with this organism before being modified for use with other species. In fact studies with *C. reinhardtii* have elucidated that proper codon usage and addition of endogenous sequences in transforming DNA increase transformation efficiency [87, 88]. Furthermore, studies with this

model organism have shown that stable genetic transformation in algae is highly species dependent and must be carefully selected depending on the target organism.

Chlamydomonas reinhardtii strain CC503

This particular lab strain of *C. reinhardtii* is the organism we will be using for this study. It is attractive because its genome is sequenced and annotated and it is cell wall deficient which allows easier incorporation of foreign genes into the host cell [89]. It is a mutant derived from strain CC-125 and it is widely used in genetic algal research [89, 90].

Chapter 3: Molecular Biology and Approaches to Algal Genetic Engineering.

In this chapter we will discuss the various methods and tools we use in order to make genetic engineering possible. In order to achieve expression and stable incorporation of a foreign gene into an algal species we employ the use of circular bits of DNA called plasmids. These plasmids contain genetic sequences along with adequate promoters and enhancers which recruit the host cells' replication machinery to produce a novel protein. In order to produce these plasmids we use an array of biological tools that allow us to accurately and precisely produce gene sequences of our choosing. Lastly, we will be describing the mechanism of action behind these biotechnological processes and giving an overview of algal gene incorporation techniques.

3.1 A Closer look at Plasmids

Plasmids are small circular pieces of double stranded DNA native to bacterial cells that have become the standard method of delivering transforming DNA to organisms. They are a ubiquitous tool in molecular biology as within their short sequences they contain replicons which are units of DNA that recruit transcription machinery autonomously [91].

Generally synthetic plasmids, called vectors, are a tool used along with molecular cloning techniques in order to construct a cascade for expression [92].

Aside from being great vehicles for the natural transfer and artificial transformation of genes, they also provide a means of genetic storage. When a plasmid is stored "naked" in solution for a prolonged amount of time it tends to degrade and denature due to shearing forces [93]; effectively destroying any information found within its sequence. However, when these plasmids are transformed into a cryogenically suitable host such as *E. coli* they can be stored for much longer periods of time and revived at regular intervals [94].

3.2 Polymerase Chain Reaction (PCR)

Perhaps the most widely used tool for generating specific DNA in biotechnology research is PCR. PCR is a method of enzyme mediated amplification of a specific sequence that can be used as a method of nucleotide addition and deletion, sequence detection, mutation, and nucleic acid generation purposes [95]. Most PCR processes rely on thermal cycling

of in an effort to drive various independent thermally dependent reactions and generate a specific sequence.

Basic PCR:

Basic PCR requires a few components in order to drive the amplification of a DNA sequence. Aside from a thermal cycler and adequate reaction vessels appropriate amounts of reaction components must be included. The most important component is a thermo-stable polymerase such as Taq polymerase. Next, appropriate reaction components such as deoxynucleoside triphosphates, template DNA, appropriate forward and reverse primers, water, $MgCl_2$, Tris-Cl, KCl, and other additives such as DMSO must be added[96, 97]. Finally, the reaction mix is put into a thermal cycler and the reaction is carried out.

Typically PCR programs aim to establish a program that denatures, anneals, and extents DNA in various cycles. The first step in thermal cycling is denaturing DNA; during this step the sample is heated above 90□ C, this causes any double stranded DNA to come apart into single strands. Next the temperature is lowered to a primer determined annealing temperature which allows primers to anneal onto the template at specific sites [98]. Then the

temperature is increased to the polymerase dependent extension temperature where the polymerase catalyzes the 5’ to 3’ addition of nucleotides [99]. It is important to note that during each round of thermal cycling the target sequence is generated exponentially, thus after various rounds of cycling the target sequence is the most abundant product[96].

General Mechanism Driving Polymerase Chain Reaction

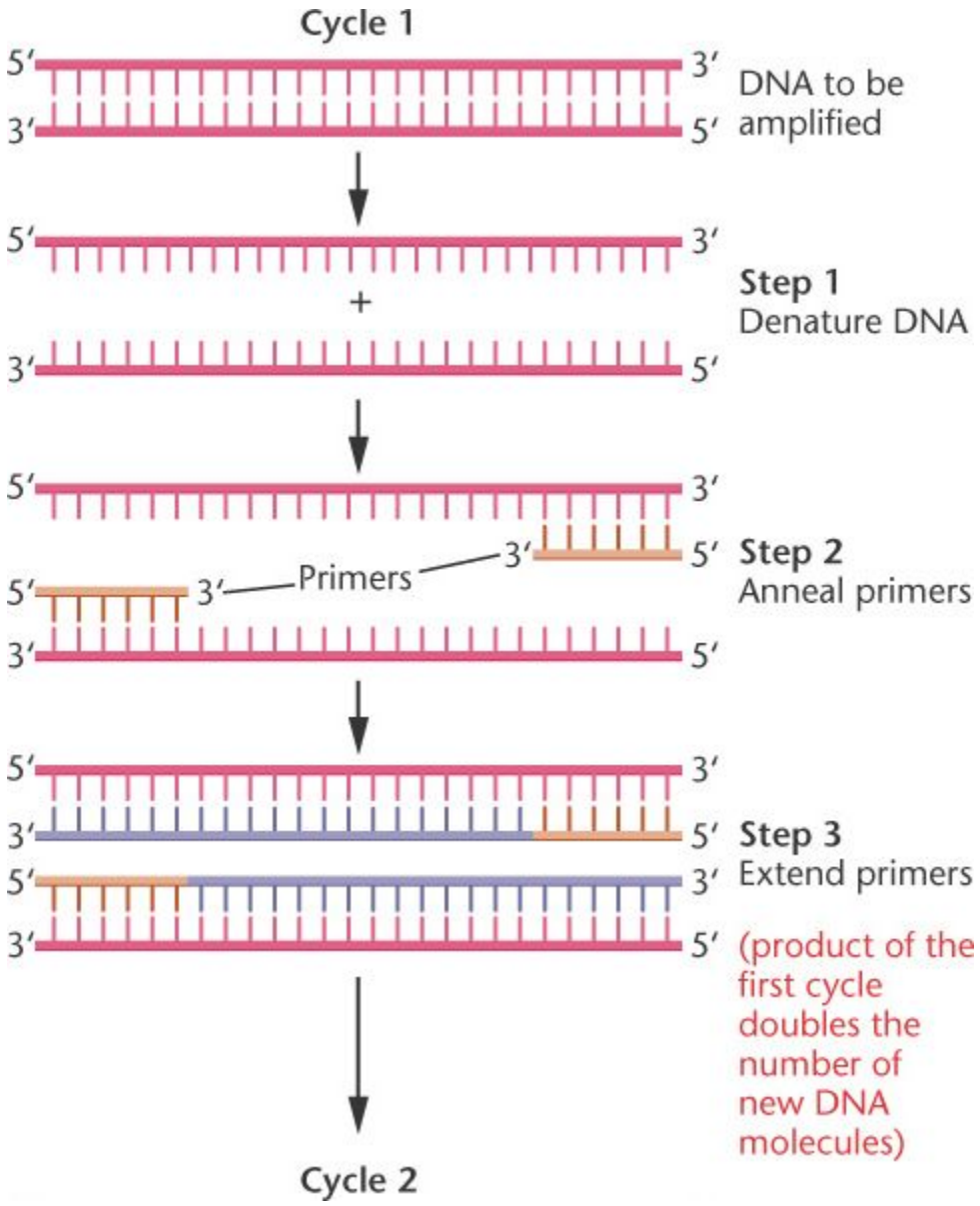

Figure 5. PCR is a process by which one specific targeted fragment of DNA is exponentially amplified. PCR is thermally cyclical process in which DNA molecules melt, anneal, and are amplified with the aid of a thermostable polymerase. Target specificity depends on the primer pair chosen. *(Image courtesy of http://bio3400.nicerweb.com/Locked/media/ch19/PCR.html)*

Reverse Transcriptase-PCR:

Basic PCR, RT-PCR uses the same thermal cycling principle in order to produce nucleic acids. However, RT-PCR is a two step process that will produce DNA from mRNA. This form of PCR is done in two separate reactions the first of which employs the use of a reverse transcriptase, a universal primer, and mRNA as a template [100]. The mRNA produced in vivo is polyadenylated thus we use this feature as a target for our universal primer. After the primer has bound the reverse transcriptase makes a single stranded DNA template of all available mRNA. This cDNA is then used as a template in a subsequent PCR reaction which amplifies the sequence of interest [101]. This form of PCR is commonly used for detection of mRNA expression and for production of coding sequences of genes.

3.3 Molecular Cloning

Cloning refers to the process of taking a particular gene or construct expressing a phenotype and producing a population of genetically identical organisms. In biotechnology research we achieve this through various methods; PCR and the use of plasmids/vectors being the main workhorses. Additionally, we employ the use of various other enzymes to aid us in the

transfer and processing of vector DNA into a cascade of recombinant DNA of our choice.

One set of indispensible enzymes are restriction endonucleases; these enzymes allow us to recombine genes by cutting the sequences at specific places. These enzymes were discovered in bacteria and act as a defense mechanism from invading viral DNA [102, 103]. Currently we have identified and purified a plethora of these enzymes and have over 600 commercially available; this gives researchers varying degrees of freedom when choosing the tools for molecular recombination [104]. Typically these are used to cut out large segments of circular plasmid DNA and separate fragments of interest. These enzymes can be used in vitro under the correct buffer conditions and depending on the enzyme used they can produce either blunt or ‘sticky’ end overhang.

Another equally important group of enzymes for molecular cloning are ligases. Ligases are a group of enzymes that catalyze the binding of DNA by creating a phosphodiester bond between the backbone of two DNA strands [105]. There are many kinds of ligases in vivo, however; the most commonly used one in vitro is T4 DNA ligase. This particular ligase uses

ATP to bind DNA blunt and sticky ends produced by restriction enzymes and can also bind DNA-RNA complexes [105]. This ligase however cannot bind single stranded DNA.

Nucleic Acid Imaging and Analysis:

While the cloning process might seem simple it actually takes a lot of time and effort because PCR is very sensitive and getting targets on the first time is rare. In fact at every point during the restriction enzyme digestion process separation of different strands of DNA in solution is necessary; thus, we employ the use of agarose gel electrophoresis as a method of nucleic acid separation.

During gel electrophoresis nucleic acid molecules are separated by size. Electrophoresis takes advantage of the negative charge of DNA and applies an electric field to drive molecules through an agarose membrane where smaller molecules travel through faster [106]. Biophysically speaking larger molecules migrate sluggishly down the matrix because they encounter more steric hindrance and collision with agarose molecules [107, 108]. These properties of agarose gels allow distinct bands to form each corresponding to a discrete sequence length.

Although these bands do form in a gel, DNA is invisible to the naked eye, thus we employ a few tools to visualize and analyze it. The most common DNA staining tool for agarose gels is ethidium bromide. Ethidium bromide is a fluorescent dye which intercalates itself between planar bases of DNA. Once DNA has been stained with ethidium bromide exposure to UV light will emit a bright band at about 600 nm which allows easy identification using the appropriate imaging material [106]. It should be noted however that linear and circular DNA have different migration patterns, mainly due to circular DNA's tendency to super coil [109].

After imaging, excising, and extracting DNA from gel fragments it is important to check that the DNA sequence is correct and intact. We can confirm our nucleotide sequence from a purified template through Sanger sequencing. These tools are used extensively during molecular cloning as a means of detection, separation, and confirmation of correct nucleic acid sequences. Only after various rounds of detection and confirmation can transformation of a designed construct be considered.

3.4 Nucleic Acid Extraction from Algal Biomass

Nucleic acid extraction is a key procedure when doing molecular cloning or attempting genetic engineering. There are a plethora of reasons why extracting organisms genomic DNA is useful; it allows us to target specific genes for over expression, detect foreign incorporated genes, and allows us to elucidate an organisms' phylogenetic background. Extracting DNA requires lysing the cell and inactivating any DNases which will destroy DNA integrity [110].

There are multiple methods of cell lysis however, as discussed above, care must be taken to ensure that full genomic DNA is not destroyed in the extraction process. This takes diligence and use of various chemical and physical means of DNA protection. First it is important to mention that DNA is a very stable molecule, upon heating it dissociates and quickly re-associates as temperature drops [111]. This property is very beneficial as other biomolecules such as RNA degrade upon heating or do not re-associate into active forms as quickly, like proteins. Another important property of DNA is that it is highly soluble in water; thus liquid extraction systems can use this quality to separate miscible and immiscible biomolecules. Lastly, it is important to note that DNases and other DNA degrading proteins use

divalent ions as cofactors for activity; therefore adding chelating agents sequester the ions and renders the proteins inactive [112].

DNA extraction:

There are a few methods of algal DNA extraction the most extensively used being the liquid-liquid chloroform-phenol extraction. This extraction method relies on extracted materials solubility difference in aqueous and organic solvents. First cells are lysed and homogenized; subsequently equal parts of a chloroform: phenol: isoamyl-alcohol solution are added to the lysate. The chloroform and phenol in the extraction solvent act as organic phases while the cell lysate acts as an aqueous phase; Isoamyl alcohol is an antifoaming agent. After centrifugation, lipids and proteins to concentrate to the organic phase and nucleic acids remain in the aqueous phase thus allowing separation of nucleic acids with minimal degradation by DNases [113]. Extraction by this method generally gives the purest longest lasting DNA.

Another less commonly used but simpler and quicker method is water or chelating resin solution extraction. In this process we use either deionized water or a 6% w/v chellex-100 resin is used as the extraction media. Cells

are lysed through thermal treatment which serves to break cell membranes and denature enzymes that will destroy genomic DNA. It should be noted that chelating agents in the chellex solution allow further inactivation of nucleases and help in the lysing process; in general, the chellex method is more effective at DNA extraction than water alone [114]. Lastly, while this is a quick and easy way of genomic DNA extraction the extract will not be pure; it will contain proteins and nucleases which will degrade DNA over time. There nucleic acids extracted in by this method must be used immediately.

RNA extraction:

Unlike DNA, RNA is not as stable and degrades much more easily. This is because RNA has an extra hydroxyl group bound to the 2' carbon position which under stressing factors such as heating or agitation can break the phosphodiester bond at the 3' carbon position resulting it degradation [115]. Additionally, RNases are ubiquitous in all organisms and very stable proteins present essentially everywhere, thus great care must be taken to ensure all lab ware is RNase free.

The presence of RNases poses a significant problem in RNA extraction as it must be performed under strict conditions in an effort to

minimize RNA degradation. We employ thermal and chemical methods to aid us in RNA purification; some of these methods include keeping samples on ice do retard enzyme activity, employing the use denaturing agents such as β-mercaptoethanol and SDS, and using RNase inhibitors. Lastly, RNA extraction from algae is harder because of the presence of a cell wall [116]; thus, we must homogenize a crude cell lysate and flash freeze it with liquid nitrogen to avoid RNA degradation. Like in DNA extraction, we use a combination of organic solvents mixed with water and rely on phase separation in order to extract and purify RNA. Furthermore, many downstream applications require RNA to be pure, thus we can use DNase 1 to purify our RNA of contaminating DNA. Lastly, other methods exist for plant RNA extraction, these are usually kits sold by large companies which use membrane binding and elution with an array of different buffers for RNA purification.

3.5 Transformation Techniques

Genetic transformation is a method that incorporates foreign genes into an organisms' genome. There are various forms of genetic transformation techniques that can be applied on various organisms. There are thermal, mechanical, electrical, biological, and even biolistic processes

that accomplish this goal. Genetic transformation can be accomplished through various methods however not all methods are efficient or even effective for all organisms. For example *E. coli* can be chemically treated with ice cold $CaCl_2$ and then transformed via heat shock but this method will not work for mammalian cells [117]. Specifically, in algae, genetic material must penetrate rigid structures such as the cell wall and along with penetrating the cell membrane. The presence for of a cell wall renders methods such as Lipofectamine transfection and heat shock useless for algal transformation [116]; thus we turn to other methods to accomplish foreign gene delivery. It is also important to note that in order to increase transformation efficiency many times we employ and enzymatic breakdown of the cell wall before attempting transformation [118]. In this section we will talk about methods that work efficiently for algal transformation.

Transformation using Shearing Particles:

In this method particles are used to shear algal cells and weaken cell walls. These can range from micron sized silicon carbide particles to macro particles such as glass beads [119, 120]. Specifically glass beads are added to a cell pellet along with a mix of transforming DNA and PEG. The sample is then vortexed vigorously and the glass beads shear algal cell walls and

membranes allowing exogenous DNA to penetrate the cell. The naked DNA is then incorporated in to the cells genome at random although it should be noted that silencing of foreign genes in algae reduces expression [121].

Transformation via Biolistic Methods:

As discussed earlier algae contain rigid cell walls that present an obstacle when trying to introduce foreign genes. In order to penetrate this rigid cell structure it must be degraded, weakened, or penetrated. Biolistic gene delivery is an efficient from of algal transformation as it allows penetration of the cell wall without prior treatment [119]. Specifically, DNA is chemically attached to gold micro particles and then shot at a solid algal culture. The particles rip through the cell walls of algae and allow the delivery of exogenous DNA into the cell in an efficient manner [122]. The only drawback of this method is the cost of the reagents and the fact that algae must survive and recover from damage to their membrane.

Transformation via Biologics:

One of the most efficient strategies for gene delivery and expression in algae is done through the use of other organisms or viruses. One facet of this method involves engineering a viral particle and incorporating

transforming DNA. The algae is then exposed to the virus thus transferring the genetic information into the host cell [119]. Another very similar facet of this method involves the use of *Argobacterium tumefaciens*. This organism is a gram-negative soil bacteria which can insert a small segment of plasmid DNA into plant cells [123]. This method also requires the genetic engineering of the bacterial plasmid and incorporation into the organism, followed by exposure to algae. Although this method of genetic incorporation is efficient and effective drawbacks include having to produce this DNA and incorporating it into the vector organism.

Transformation via Electroporation:

Electroporation presents a quick, efficient, cheap, and reproducible method for the transformation of algae [119]. In this method high voltages are applied to cells directly in pulses over a small surface area. The high voltage causes a stark difference in electrical potential across the cell membrane allowing it to temporarily become porous. Negatively charged DNA then enters into the positively charged cytosol and incorporates itself into the organisms' genome. This method is quick and effective however a very high cell density and a specific cell physiology are required [124]. Additionally, since high voltages are used rampant cell death is expected

[125]. This technique was used in the algal transformations discussed in this study.

3.6 Transformant Selection Methods

Once transformation has been performed a thorough screening must be done in order to make sure proper gene incorporation has occurred. Fortunately, we have various methods for the screening and confirming that exogenous DNA has been successfully incorporated into the cell and that it is being expressed. One of the oldest selection and most used markers is antibiotic resistance. Many organisms across kingdoms, particularly bacteria, have developed antibiotic resistance genes which allow them to survive under antibiotic pressure [126]. These genes are extracted from host organism and transformed into various other organisms. The expression of these genes in the transgenic species confers antibiotic resistance over the wild type making screening via antibiotic pressure a viable way for transformant selection.

Another widely used selection system involves the use of fluorescent proteins. Animals such as the jellyfish *Aequorea victoria* are naturally bioluminescent and express proteins such as GFP which allow them to

'glow' [127]. The genes for these proteins have been extracted and expressed in other organisms successfully and expression confers the transgenic organism the ability to glow. Thus, under the right treatment conditions this can be used as a selection marker for positive transformants as wild type strains will not glow when subjected to the proper excitation wavelength. Additionally, GFP is usually employed as a fusion protein in order to elucidate the cellular localization of other proteins [128].

3.7 Challenges with Algal Transformation

Algal transformation is difficult for various reasons; the most simple being the presence of a cell wall which makes introducing foreign DNA challenging. Beyond the presence of this plant organelle algae have a tendency to silence transgenes. Gene silencing happens due to the local environment of where the gene is located; specifically, DNA associated proteins such as histones and other chromatin components are highly dynamic [129]. This dynamic nature of chromatin causes tight packing of histones which results in low expression or complete silencing of transgenes.

Methods that employ enzymes which aid in the unpacking of chromatin near transgene location have been employed in mammalian

systems and have resulted in increased expression. However, there methods have not been developed for algal systems to date. Instead, researchers have turned to using strong endogenous promoters in an effort to have the algal cellular machinery localize to the region of interest. Additionally, they have included introns from native promoter and enhancer regions in the expression cassette that have increased transgene expression [130].

Gene expression is tightly regulated in all organisms according to their metabolic quotas or in response to stimuli. This regulation is mediated via replication machinery and chromatin modifying enzymes. Furthermore, epigenetic factors such as DNA methylation and histone modifications contribute to native gene expression, thus having naked foreign DNA causes expression to be highly dependent on where it is integrated in the genome [129].

Chapter 4: Design of Vector Systems for Foreign Gene Expression of BCL-XL and IRT2 in *Chlamydomonas reinhardtii.*

Algae is a popular source of biomass for the biotech industry. This biomass is industrially attractive because, as discussed earlier, it is cheap and quick to generate and its production presents an opportunity for bioremediation. In recent years, the industrial scope has zoomed into the strain *C. reinhardtii* as it has been used widely as a model organism for algal genetic engineering. This quality is attractive as it allows researchers to induce physiological and metabolic changes via genetic engineering thus labeling this strain as a potential candidate for industrial biomass production.

Genetic engineering of algal cells is challenging and requires molecular systems and transformation methods that allow the penetration and expression of exogenous DNA. As discussed earlier, we employ the use of strong endogenous promoters and include native intronic sequences within the expression cassette in an effort to increase expression. We aim to create stable transformants that do not silence transgenes even after many rounds of cell division. Furthermore, we aim to achieve recombinant protein expression in order produce a better strain in to improve industrial productivity.

In this study, two genes are selected and incorporated into an expression cassette in a twofold effort to increase algal stress tolerance and to increase intracellular iron concentrations. The first gene, BCL-XL, aims to increase algal stress tolerance to apoptosis. The second gene, IRT2, aims to increase intracellular iron concentrations via over expression in an effort to create a viable algal strain for magnetophoresis.

4.1 The Role of BCL-XL in Apoptosis

Apoptosis is a mechanism of programmed cell death (PCD) that occurs in response to environmental stress. It is a highly regulated process that developed in order to preserve a healthy community of cells by sacrificing cells that have undergone irreversible cellular damage [131]. This process ensures that cells containing genetic mutations do not propagate and cause a mutant cell lineage. Apoptosis is induced by physical, chemical, and biological means that cause DNA damage or intracellular flaws. Once the cell recognizes the internal damage it will employ either an intrinsic or extrinsic apoptosis pathway resulting in cell death [131]. PCD is a well studied phenomenon which is characterized by distinct morphological changes such as cell shrinkage, nuclear, chromosomal and DNA fragmentation and blebbing [132]. Unlike necrosis, PCD serves to keep a

cell population healthy by preventing harmful intracellular components from dying cells to be released into the environment.

Apoptosis is a caspase mediated process that employs various different cofactors and regulatory proteins as it is a direct cause to many serious degenerative diseases like cancer. At many points in the pathway there are regulatory points that either promote or inhibit apoptosis. We will focus on BCL-2 family proteins encoded by the human BCL2L1 gene as they serve as strong mediators of apoptosis. The BCL2L1 gene is human gene that encodes pro-apoptotic BCL-XS and anti-apoptotic BCL-XL through alternative splicing. BCL-XL (B-cell Lymphoma Extra Large) was found in B-cell lymphoma tumors and is a strong inhibitor of apoptosis across many domains of life [133]. Although the exact apoptosis inhibition mechanism has not been elucidated it is thought to prevent the formation of the permeability transition pore in the mitochondrial membrane thus preventing the release of cytochrome-C into the cytosol [134]. In this study, this gene was transformed into *C. reinhardtii* in an effort to confer better stress tolerance over the wild type cells.

Diagram of Apoptosis Pathway

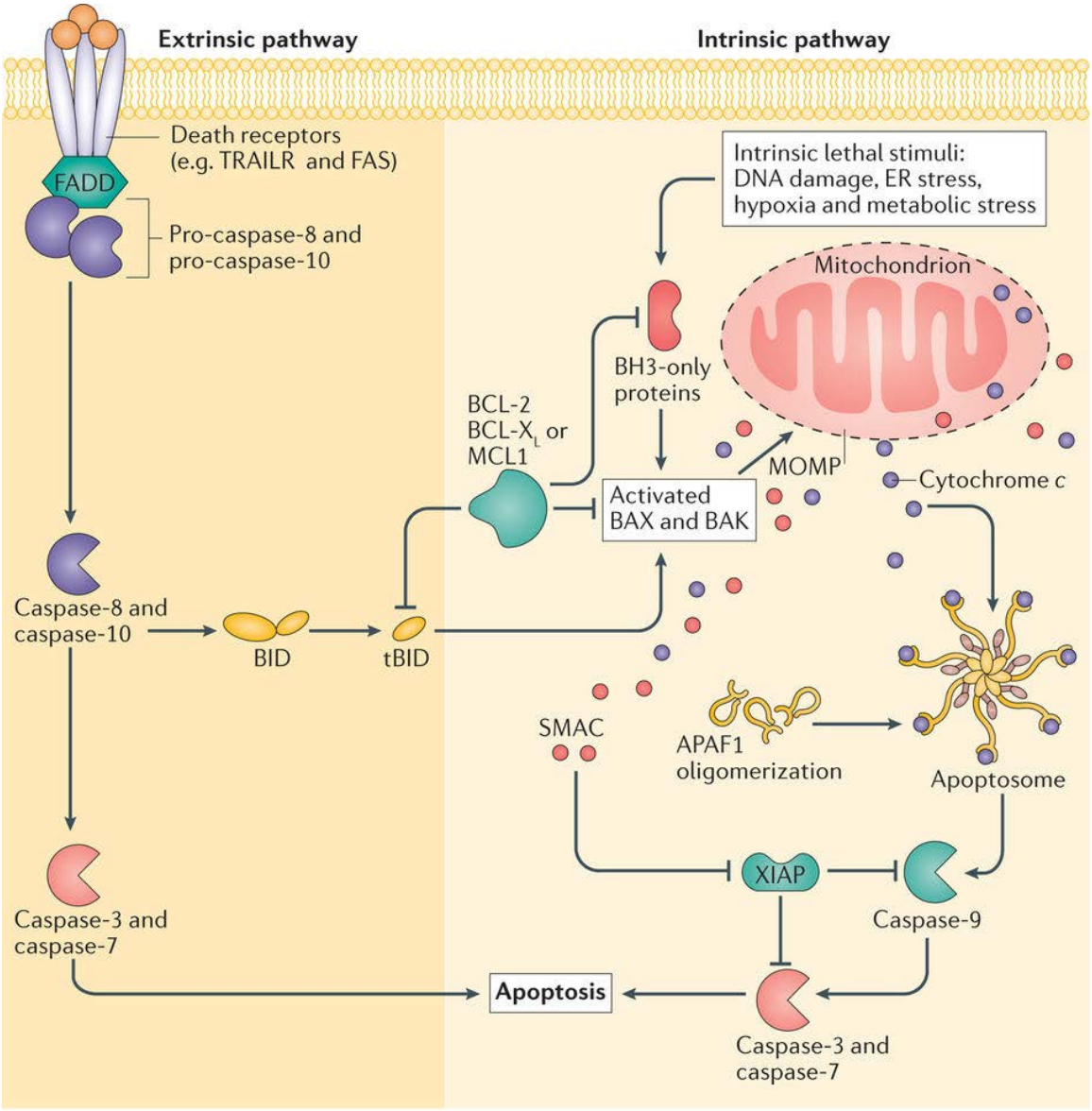

Figure 6. This diagram shows a simplified overview of cellular apoptosis pathway. Apoptosis can induced via an extrinsic or intrinsic pathway depending on stress and culture conditions. Notice how BCL-XL is an apoptotic inhibitor for both pathways; this is why this protein was chosen for expression [145].

4.2 The Role of IRT2 in Iron Metabolism

Aside from being a model organism for genetic engineering and other academic studies *C. reinhardtii* serves as a model for iron nutrition and uptake pathway studies in plants. Their fast growth rate provides an experimental system for the understanding of plant iron metabolism at the cellular level. Through extensive studies researchers have noticed that various metabolic and physiological shifts occur in the cell depending on extracellular and environmental iron concentrations [135]. Additionally, it has been discovered that photosynthetic performance is affected by iron supply to the cell; thus in an effort to increase industrial productivity it is important to understand the underlying mechanistic factors at play within the iron metabolism.

Although the natural habitat of *C. reinhardtii* and other land plants is quite similar it must be noted that the most common land dwelling ancestor, *Arabidopsis* ,split nearly 700 million years ago [136]. Even after years of independent evolution however, both organisms still have highly conserved photosynthetic and iron assimilation machinery. In plants, two organelles; the mitochondrion and the chloroplasts, require iron as an important cofactor for producing cellular energy either via photosynthesis or respiration [137].

Furthermore, both organelles house various iron dependent proteins that aid in the electron transfer pathways, thus making iron a vital component for cellular energy production [137]. Moreover, iron takes an important role as it is a cofactor for other metalloproteins which are involved in fatty acid metabolism, amino acid synthesis, DNA replication and repair, and reactive oxygen species inactivation.

Iron is a vital component for plant cellular processes as it is an important cofactor for metalloproteins needed for cellular fitness and survival. Additionally, it has been studied that *C. reinhardtii* has no mechanisms for iron export and that under iron excess conditions it tends to hyper-accumulate iron in the cytosol [135]. Both of these physiological characteristics present an opportunity for genetic engineering; specifically by over expressing passive membrane bound iron transporters such as IRT2. IRT2 is a native membrane bound protein found in *C. reinhardtii* that is involved in the passive transport of ferrous iron [138]. This protein is a ZIP family protein that is up-regulated during iron deficiency thus pointing it as an iron transporter [139]. The goal of over expressing this protein is to increase the intracellular concentrations of iron in an effort make a viable strain for magnetophoresis [140]. One possible drawback of iron

accumulation is cell toxicity, however evidence suggests that *C. reinhardtii*, like other land plants, concentrates excess divalent ions in acidic membrane bound vacuoles called acidocalcisomes [141]. Therefore, by exposing transgenic cells to high concentrations of chelated ferrous iron media we hope to hyper accumulate intracellular iron in an effort to make magnetophoresis feasible.

Iron Assimilation Pathway in *Chlamydomonas reinhardtii*

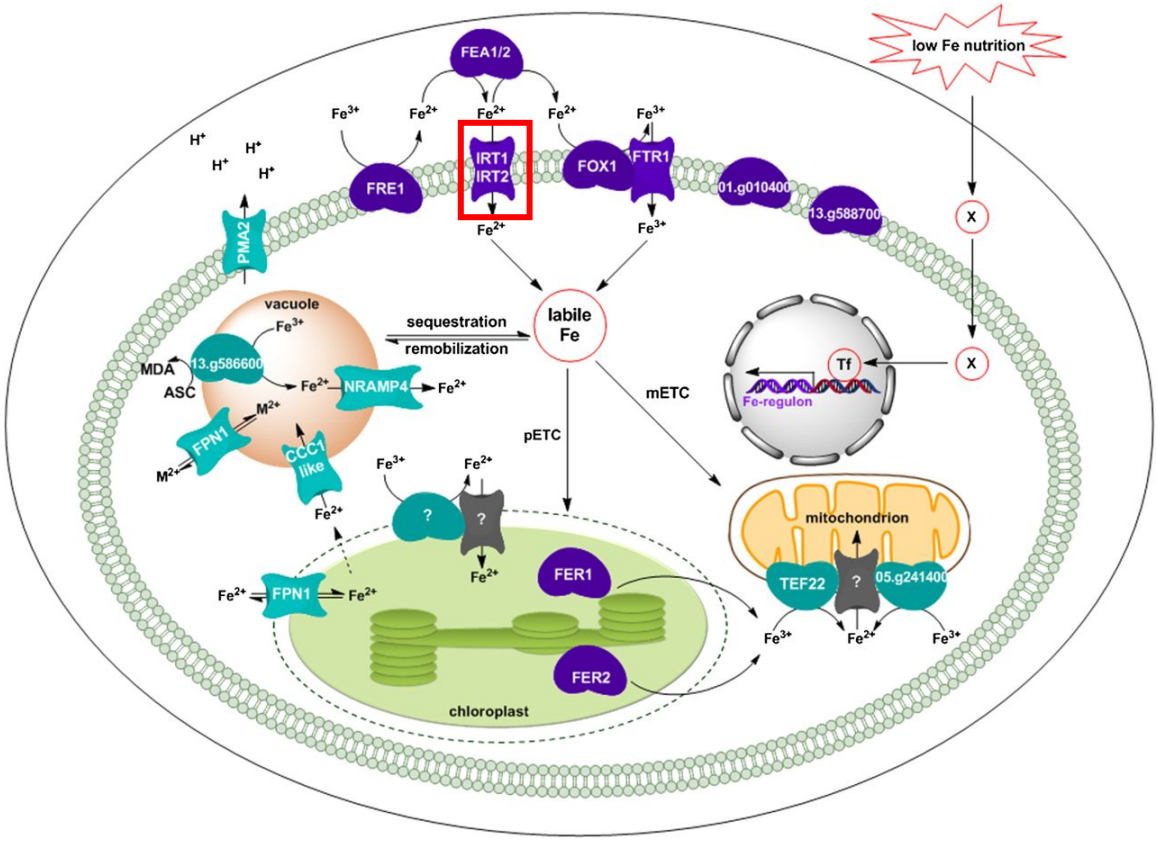

Figure 7. This diagram shows a simplified schematic of the iron assimilation pathway present in *C. reinhardtii*. Notice all the iron transporters in the plasma membrane. All of these are potential targets for over expression. In this study IRT2 (red box) was targeted as it is a passive ferrous iron transporter [146].

4.3 Construction of Vector systems for Transformation

In this section we will discuss the methods used for the construction of vectors containing the appropriate genes within the expression cassette. As a starting point we acquired a vector (pSBP-Hyg) used in the transformation of Chlamydomonas species from the Miller Lab at UMBC; this vector contains the HSP70A-RBCS2 tandem endogenous promoter coupled with the RBCS-Beta2-Tub promoter for expression of the foreign gene and the antibiotic resistance gene [147]. Additionally, it contains intronic sequences from the RuBS sequence in the expression cassette along with the 3' untranslated region flanking a eukaryotic antibiotic resistance marker [147]. Furthermore, it contains the necessary sequences for high copy replication and selection in *E. coli.* Specifically, the bacterial features this vector contains are; a high copy origin of replication, and an antibiotic resistance gene conferring resistance to Ampicillin. It is also important to note the Spe1 and BamH1 restriction sites flanking the gene of interest as they will are used extensively in the cloning process. Algal replication features and function can be appreciated in figure 8.

Plasmid Map of SBP-pHYG

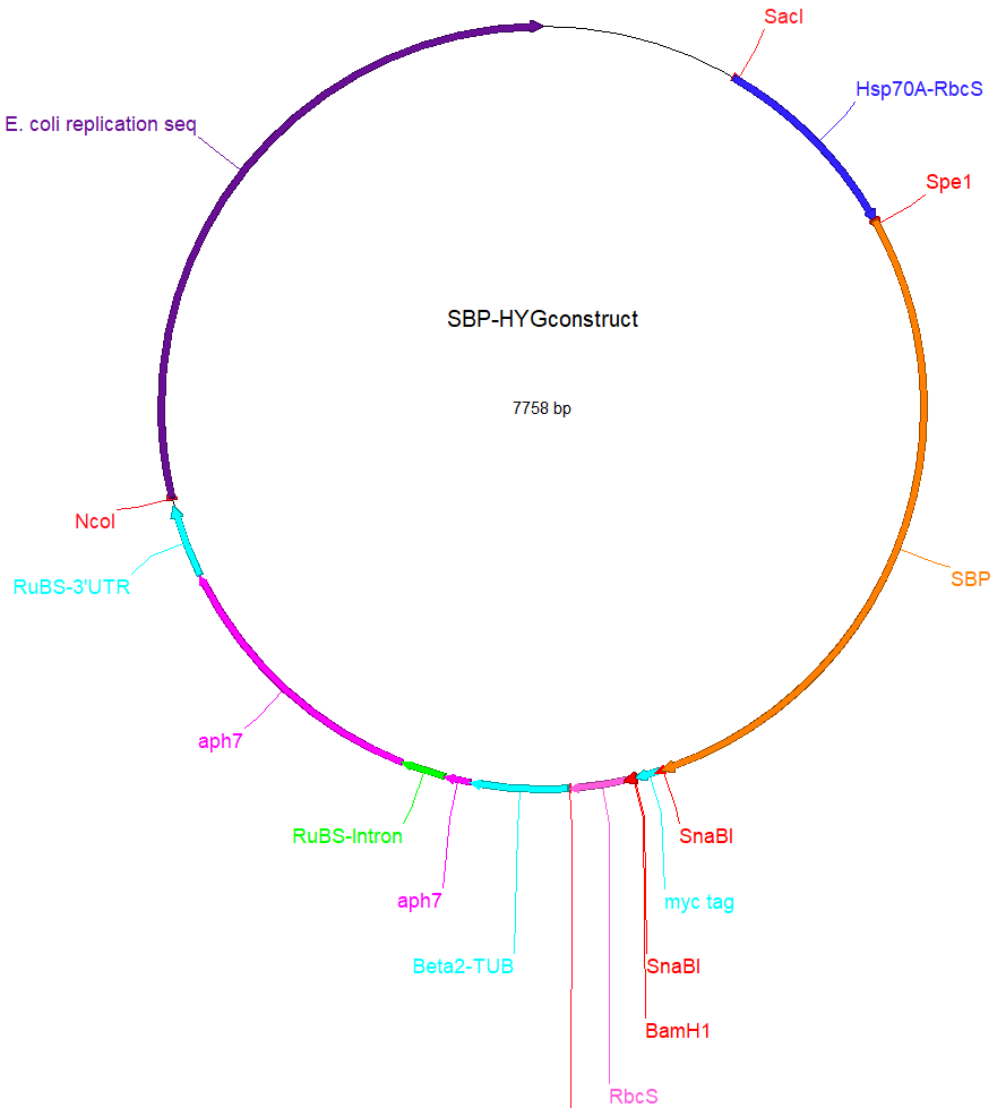

Figure 8. This is a schematic representation of the algal transformation vector used in this study. It contains 2 replicons; one for storage and propagation in *E. coli* and one for *protein* expression in *C. reinhardtii.* Notice restriction sites BamH1 and SPE1 as that is the cloning site of interest for this study.

4.3.1 Isolation and Modification of Target Genes

Isolation of BCL-XL:

The *C. reinhardtii* codon optimized sequence for BCL-XL was initially extracted from the pRelax vector constructed by Rosenberg et al. via PCR. Amplification was driven by phusion polymerase using BCL-XL_Flag_Fwd and BCL-XL_Rev primers found in table 1. The PCR conditions presented in table 2 were used to drive amplification. It should be noted that during this PCR we added an N' terminal flag epitope tag along with Spe1 and BamH1 cloning sites.

Table 1: Primer Sequences Employed to Isolate and Modify BCL-XL Gene

Primer	Sequence
BCL-XL_Rev	ggatccttaCTTCCGCGAGAACAGGCTG
BCL-XL_Flag_Fwd	actagtATGgattataaagatgatgatgataaaGGAGCCGGCT GCGCTGGT

Table 2: PCR Program Employed to Amplify BCL-XL

Step :	Temperature(C)	Time
Denaturation	98	30 s
Thermal Cycling(35 cycles)	98	10 s
	64	30 s
	72	30 s
Final Extension	72	10 min

Once PCR concluded samples were analyzed on a 1% w/v agarose gel stained with Ethidium Bromide. Upon UV analysis the gel yielded discrete bands of the appropriate length ~740 BP. This Fragment was extracted and purified via Qiagen gel extraction kit, and stored at -20 C for downstream applications.

Isolation of IRT2:

IRT2 is a native gene in *C. reinhardtii* thus the native sequence contains introns which we did not want in our expression system. Thus we decided to isolate the coding sequence from mRNA via RT-PCR. We harvested CC503-WT (wild type) via centrifugation during mid exponential phase and extracted total mRNA using the Omega E.Z.N.A Plant RNA kit. Extracted mRNA was then used as a template in an RT-PCR first strand synthesis reaction using an oligo(dT) 15 primer, the promega M-MLV reverse transcriptase and following the conditions in table 3.

Table 3: Reaction Conditions for RT-PCR First Strand Synthesis of IRT2

Step	Temperature (C)	Time
Denaturation	70	5 min
First Strand Synthesis	37	60 min

Table 4: Primers Sequences Employed for Isolation of IRT2 from cDNA

Primer	Sequence
IRT2_Fwd	CCTGGTTGTGCGGGCGTCT
IRT2_Rev	ggatccTTACGGGGCTGCTGGGGCT
IRT2_Flag_Fwd	actagtATGgattataaagatgatgatgataaaCCTGGTTGTGCGGGCGTCT

Following first strand synthesis we performed second strand synthesis using cDNA as a template and IRT2_Fwd and IRT2_Rev as primers. For this reaction we initially used a 2 step PCR using phusion polymerase run at a 72 C annealing temperature to no avail. Instead, we employed a gradient PCR protocol using the same starting materials but running the annealing temperature gradient from 67 C to 69 C with a change of 1 C; see process flow diagram in figure 9. Upon reaction termination samples were analyzed on a 1% w/v agarose gel stained with Ethidium Bromide. Upon UV analysis the gel yielded 1 discrete band of the appropriate length ~1450 BP along with other lanes showing nonspecific amplification seen in figure 10A.

The resulting DNA band was excised and purified using a Qiagen gel extraction kit; purified DNA was then used in a subsequent phusion based gradient PCR reaction with annealing temperature spanning from 66.5 to 68 C with a change of .5 C. Once reaction terminated samples were again analyzed on an equally composed agarose gel, in this second round of

gradient PCR we were able to observe stronger yielding bands at the same molecular weight as seen in figure 10B.

Following gel extraction and purification of lanes 2 and 3 in figure 10B we ran one last round of PCR this time aiming to incorporate the flag epitope tag. For this reaction we used a phusion based polymerase, and IRT2_Flag_Fwd and IRT2_rev as primers. Learning about our ideal annealing temperatures from gradient PCR we designed the program that employed this annealing temperature; 67.5 C. Once the reaction concluded we again ran the products on an agarose gel under the same conditions and got 3 discrete bands at the approximate length of ~1470 BP seen in figure 11. Since resolution on agarose gels is highly dependent on running conditions and cannot resolve very well between small fragments we purified the gel fragments and stored at -20 C for downstream applications and confirmation of epitope tag and cloning site addition.

Process Flow diagram of IRT2 Isolation and Propagation

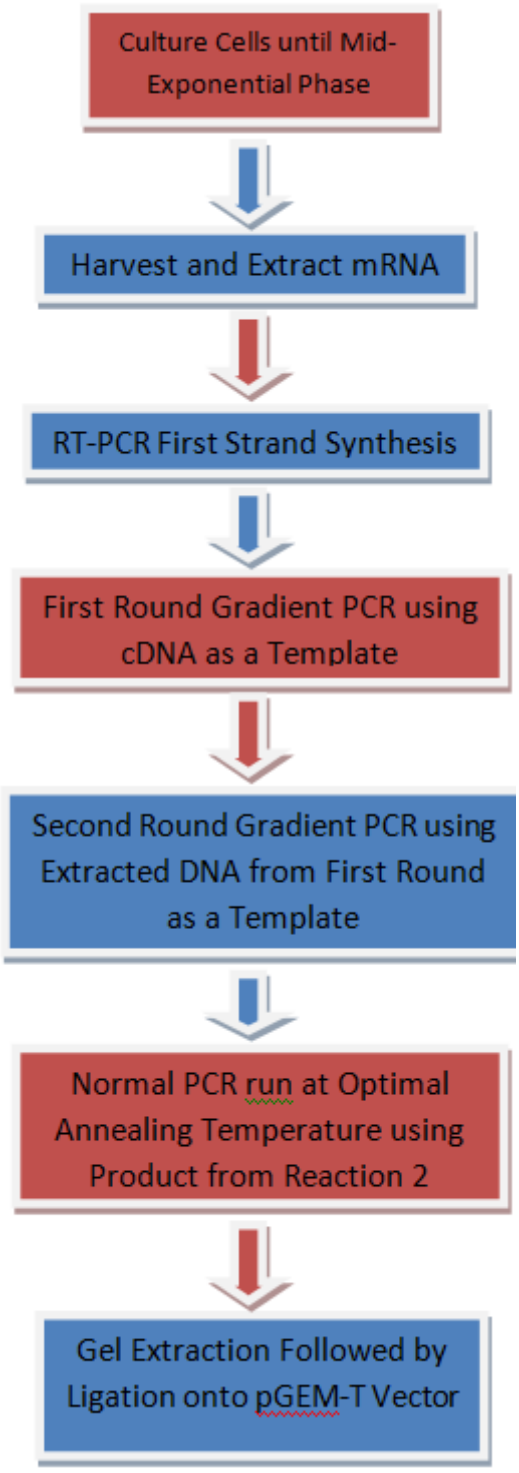

Figure 9. This image shows a process flow diagram of the steps taken to isolate IRT2 from *C. reinhardtii.* Our goal was to isolate from mRNA in order to have the coding sequence without introns included.

Agarose Gel Results of Gradient PCR's Targeting IRT2

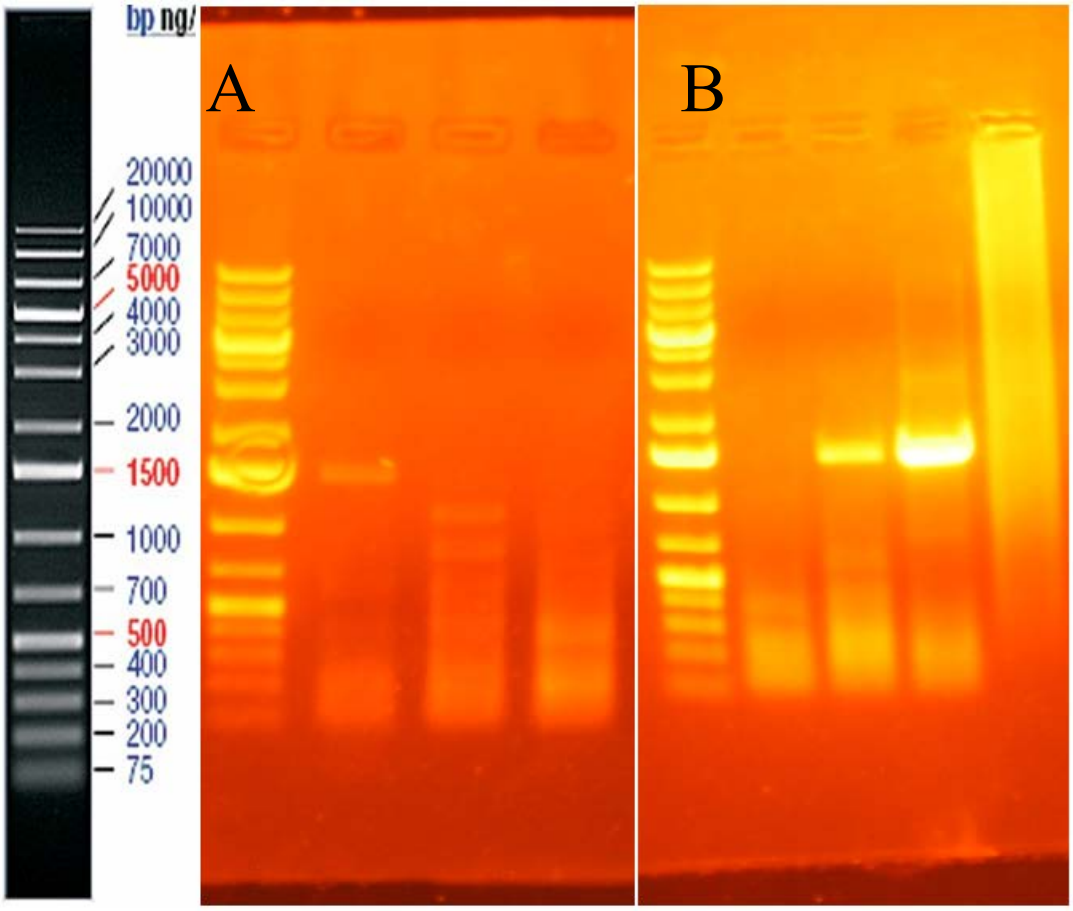

Figure 10. **A.** This image shows an agarose gel containing the products of the first gradient PCR targeting IRT2. Notice how the first lane produces a faint band at the appropriate band length of ~1450 BP. Also notice the non specific amplification that occurs at other higher temperatures. **B.** This image shows an agarose gel containing the products of the second gradient PCR targeting IRT2 using the product from the first reaction as a template. Notice how the second and third lanes produce strong bands at the appropriate band length of ~1450 BP. Also notice the non specific amplification that occurs in lane 4.

Agarose Gel Result of PCR Targeting IRT2

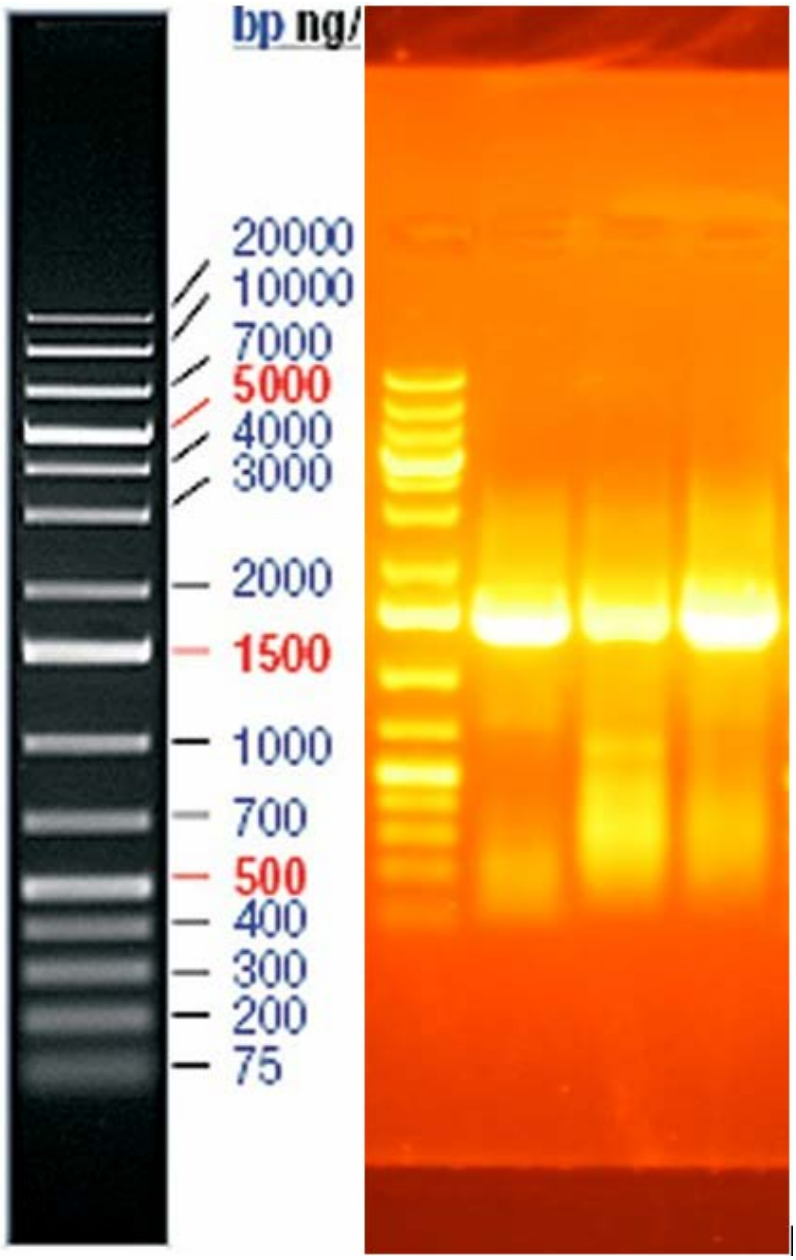

Figure 11. This image shows an agarose gel containing the products of the PCR targeting IRT2 using the product in lane 3 of the second gradient PCR as a template. Notice how the all lanes produce strong bands at the appropriate band length of ~1450 BP. Through various rounds of gradient PCR the optimum annealing temperature of 67.5 C was found, each reaction was supplemented with different DMSO concentrations.

4.3.2 Ligation onto pGEM-T for Feature Confirmation

Once both genes of interest, BCL-XL and IRT2 had been isolated via various rounds and forms of PCR we decided to confirm that all cloning sites and epitope tags had been incorporated effectively and correctly. In order to do this we employed the use of Promega's pGEM-T easy vector systems. This system was chosen because it is a commercial vector that is well annotated and contains a 3' T overhangs which allow easy and efficient ligation of blunt ended DNA sequences (produced by phusion) after an A-Tailing reaction. Additionally, this vector contains a coding region for β-galactosidase which can be used for blue/white screening of colonies following transformation.

A-Tailing Reaction with Genes of Interest:

One property of phusion DNA polymerase is that it generates blunt ended products, thus in order to ligate our genes of interest onto our pGEM-T vector we had to undergo an A-Tailing reaction. During this reaction we use Taq DNA polymerase which can leave 3' overhangs to drive our reaction to incorporate a single 3' Adenine moiety at the end of our genes of interest. To a PCR tube we added appropriate buffers, enzymes, template DNA, and only dATP; we then ran the reaction at 72 C for 20 minutes.

After completion of A-tailing reaction products were ligated according to the Promega ligation protocol using ligase provided with the kit. Reactions were incubated 4 C overnight and transformed into chemically competent DH5α *E. Coli* via heat shock. Transformed samples were cultivated in LB-Ampicillin plates treated with X-Gal and IPTG and incubated at 37 C for 16 hours. Following overnight incubation plates were recovered; many white and blue colonies were visible, plates were placed in 4 C for 1 hour to intensify blue colored colonies. After incubation, single white colonies were chosen and cultured in 10 mL of LB-Ampicillin at 37 C for 16 hours. After incubation liquid colonies were harvested and plasmids were extracted and purified using the Qiagen Plasmid miniprep kit. Plasmids were treated with appropriate stains and run on an agarose gel to confirm efficient insert integration. Results can be appreciated in figure 12, note that supercoiling is evident, thus we look at the faint middle band in both lanes 1 and 2 to estimate plasmid size; lane 1 holds BCL-XL-pGEM-T -3750 BP and lane 2 holds IRT2-pGEM-T ~4500 BP respectively.

Agarose Gel Analysis of pGEMT Plasmids

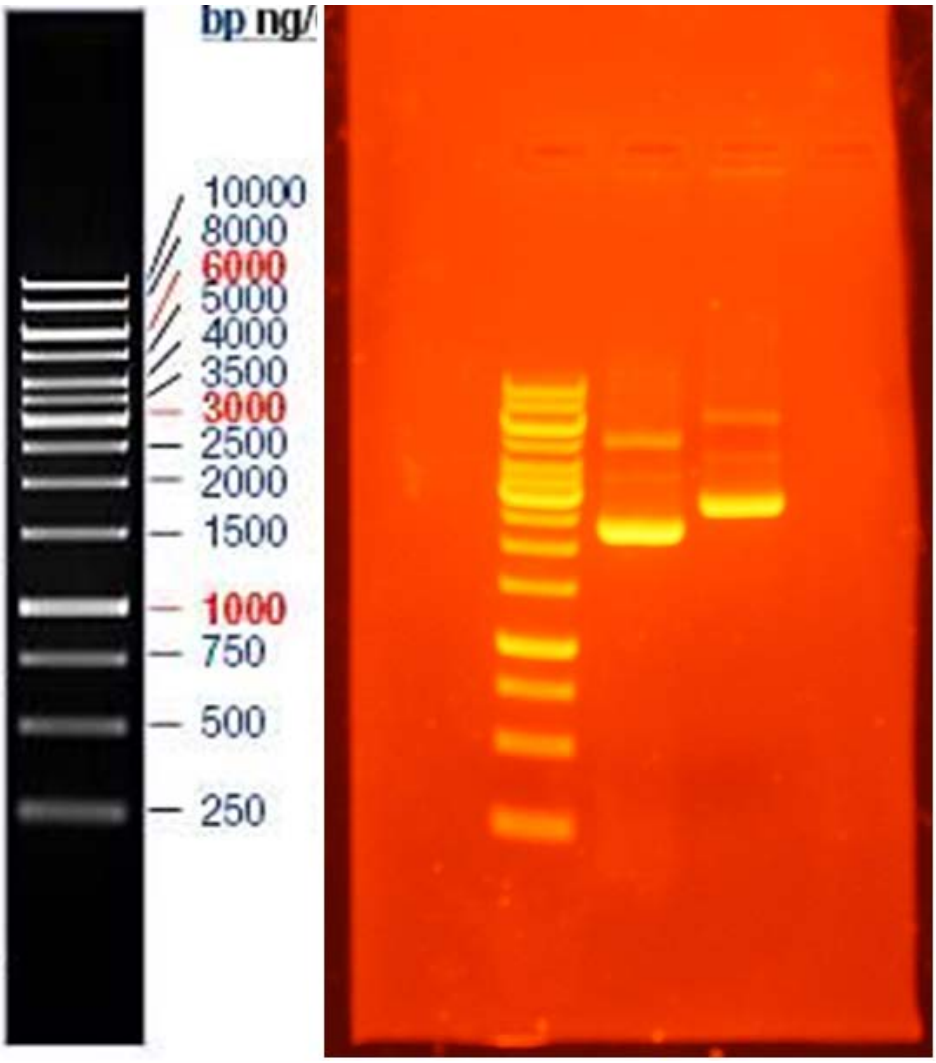

Figure 12. This image shows an agarose gel containing the products of the pGEM-T plasmids extracted from E.coli following transformation with ligation products with BCL-XL and ITR2. In lane 1 we can see the pattern left by BCL-XL-pGEM-T; this is evidence of supercoiling, we can estimate the plasmid length by the faint band in the middle ~3750 BP. In lane 1 we can see the pattern left by BCL-XL-pGEM-T; this is evidence of supercoiling, we can estimate the plasmid length by the faint band in the middle ~3750 BP. In lane 2 we can see the pattern left by IRT2-pGEM-T; this is evidence of supercoiling, we can estimate the plasmid length by the faint band in the middle ~4500 BP.

Following agarose gel analysis plasmids were sequenced to ensure proper epitope tag and cloning site addition using the primer sequences found in table 5. Once sequencing results confirmed that all relevant features had been added to the gene of interest pGEM-T plasmids were stored in -20 C for downstream use.

Table 5: Primers Employed for Sequencing p-GEM-T Constructs

Primer	Sequence
M13 Fwd	CGCCAGGGTTTTCCCAGTCACGAC
M13 Rev	TCACACAGGAAACAGCTATGAC

4.3.3 Digestion and manipulation of pSBP-HYG

Once we confirmed that our genes of interest included all the epitope tags in frame and cloning sites we desired were added appropriately we turned to isolating our vector of interest. In order to do this we first did a double digestion with pSBP-HYG using Spe1 and BamH1 after the digestion we ran it on an agarose gel and could see the characteristic pattern expected (see figure 13).

Agarose Gel Analysis of SBP-pHYG Double Digestion

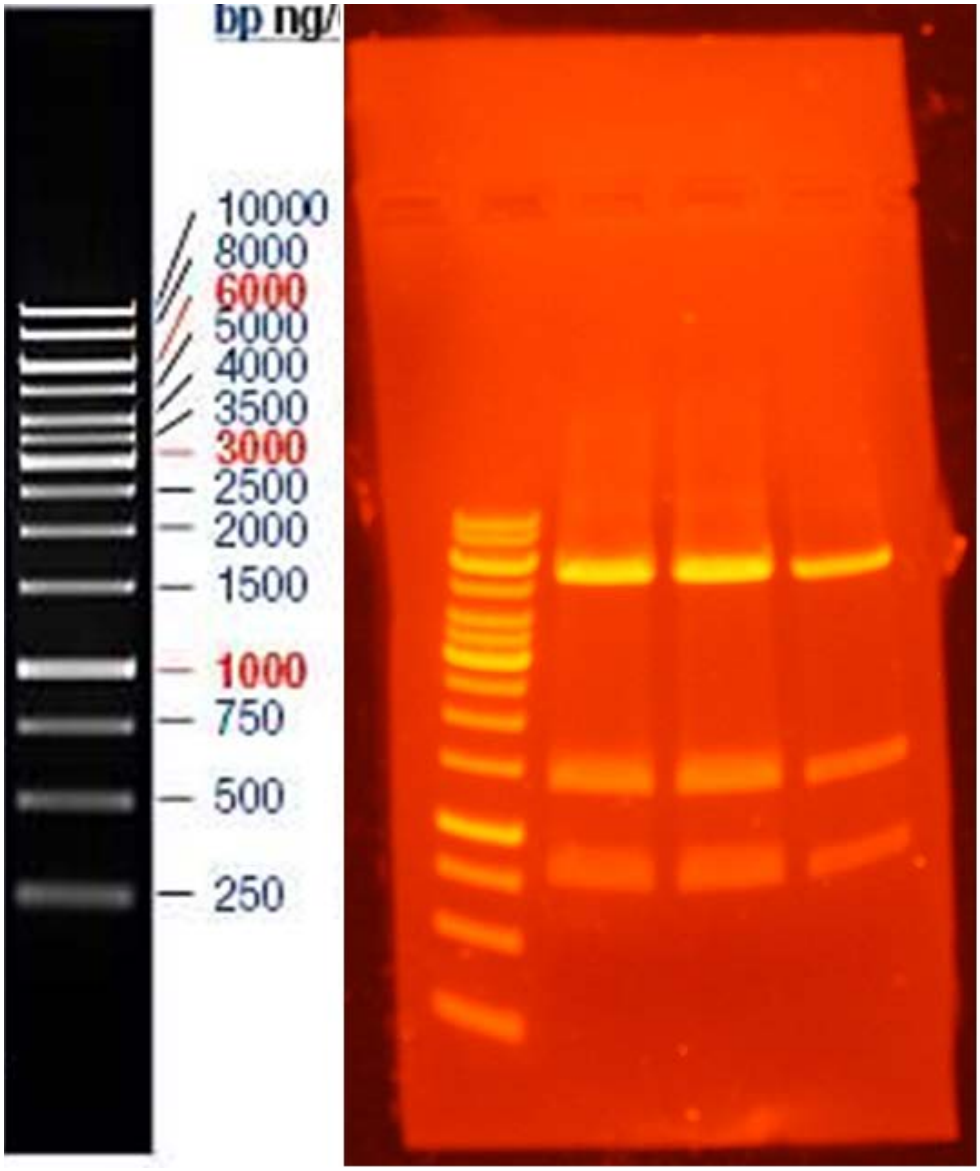

Figure 13. This image shows an agarose gel containing the products of a restriction enzyme digestion of the SBP-pHYG vector. This plasmid was digested using Spe1 and BamH1 in cutsmart buffer. All lanes contain the same band patter with the empty pHYG vector at ~5700BP.

Next we excised and purified the gel fragment and decided to compare the double digest efficiency by running it next to a single digest and the intact plasmid as an absolute control. The results can be appreciated in figure 14 where lane 1 is the double digest yielding a band of expected size ~5500 BP, lane 2 is the single digest giving a band of expected size ~7760 BP, and the third lane showing the bands typical of a supercoiled plasmid (lanes 4 and 5 are duplicates of lane 2 and 3 respectively). Notice how the first lane yields a band smaller in molecular weight than both lanes 2 and 4, indicating that our double digest did in fact work. Once this result was obtained lane 1 was excised, purified by the same previous methods and stored at – 20 C for later use.

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Agarose Gel Analysis of SBP-pHYG Double vs. Single Digest

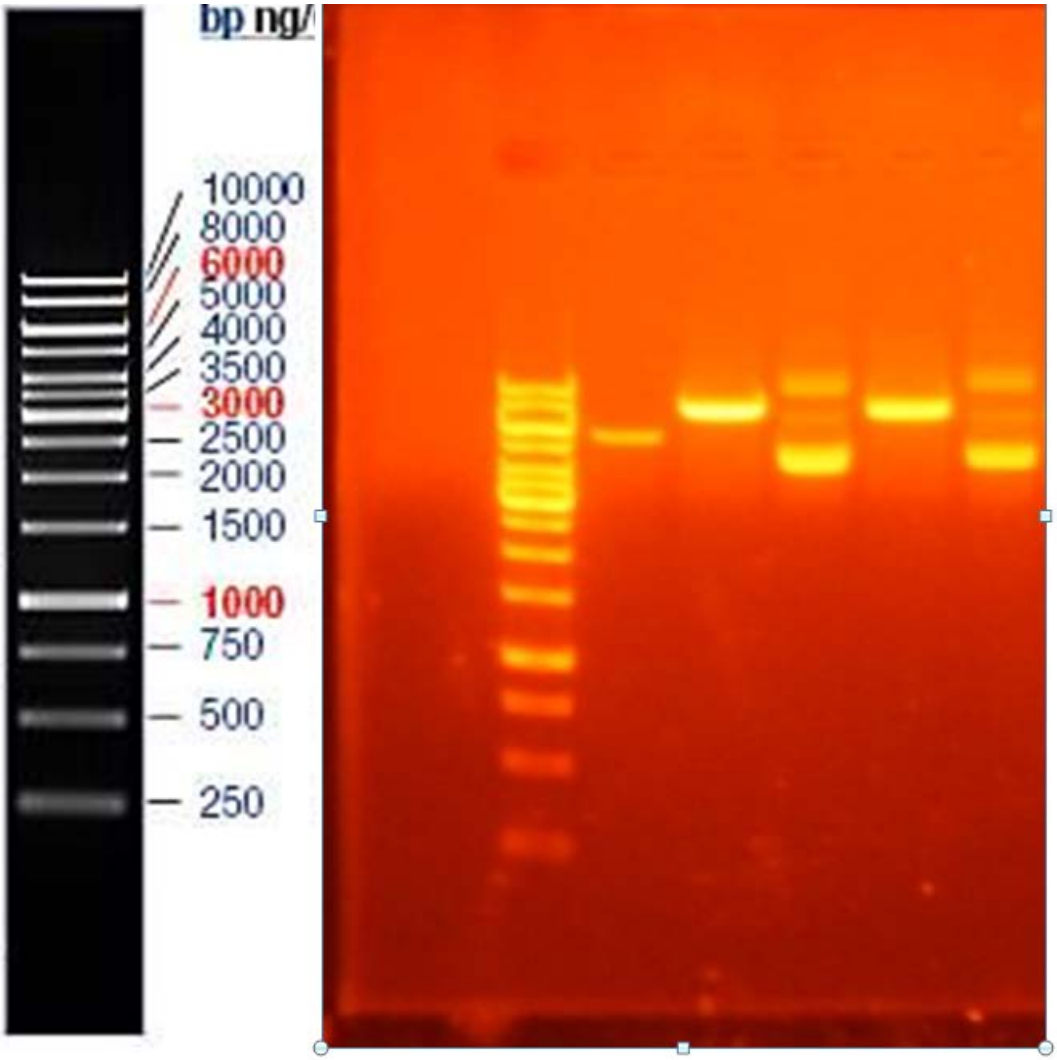

Figure 14. This image shows an agarose gel comparing the products of a restriction enzyme single digestion of the SBP-pHYG vector and the purified product of interest from the double digest. The first lane contains the empty pHYG vector at ~5700BP, lanes 2 and 4 contain the linearized SBP-pHYG vector, and lanes 3 and 5 contain the uncut supercoiled SBP-pHYG vector. This image confirms our double digest was successful.

4.3.4 Digestion of pGEM-T Vectors and subsequent ligation of Gene of Interest to Expression Vector

Once we obtained the linearized empty pHYG vector including sticky ends left by digestion with Spe1 and BamH1 we decided to digest our pGEM-T vectors containing our genes of interest for subsequent ligation. In the interest of making our downstream ligation more efficient we also digested the pGEM-T vectors with BamH1 and Spe1. Upon digestion and analysis of these vectors we obtained the expected lanes of BCL-XL (~740 BP) and IRT2 (~1470 BP) which were excised purified and then used in a ligation reaction.

After purification of both fragments we used T4 DNA Ligase to drive our ligation reaction; we made two different reaction regimes with vector: insert molar ratios of 1:1 and 1:3. Once mixed we let our reaction incubate overnight at 4 C and then used our ligation products and transformed into chemically competent DH5α *E. Coli* via heat shock. Transformed samples were cultivated on LB-Ampicillin plates and incubated at 37 C for 16 hours. Following overnight incubation plates were recovered; many individual colonies were visible. Subsequently 3 single colonies were chosen and individually cultured in 10 mL of LB-Ampicillin at 37 C for 16 hours. After

incubation liquid cultures were harvested and plasmids were extracted and purified using the Qiagen Plasmid miniprep kit.

After miniprep plasmid purification we wanted to check whether or not our gene of interest had been appropriately ligated onto our vector; thus we confirmed via digestion of our plasmid. Specifically, we digested 2 out of 3 of our recovered plasmid using Spe1. For this we used the empty linearized pHYG vector as a control along with the uncut purified plasmid. Results of this digestion are shown in figure 15 where lane 1 shows the empty linearized pHYG vector showing a band around ~5500 BP, lanes 2 and 4 show the linearized IRT2-pHYG showing a bands around ~6950 BP, lanes 3 and 5 showing the uncut supercoiled IRT-pHYG, lanes 6 and 8 showing the linearized BCL-XL-pHYG giving bands around ~ 6220 BP, and lanes 7 and 9 showing the uncut supercoiled BCL-XL-pHYG. Upon this confirmation we saved the purified constructs for downstream transformation. The vector maps for both of these constructs can be appreciated in figure 16 and figure 17.

Agarose Gel Results of IRT2 and BCL-XL ligation onto pHYG Vector

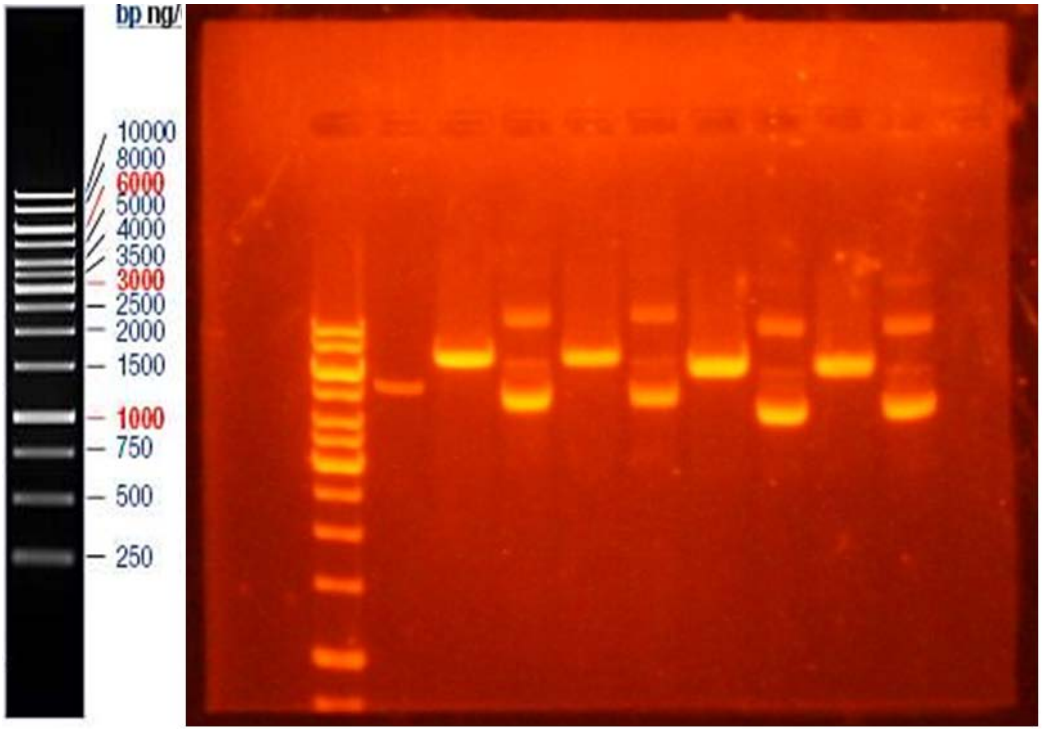

Figure 15. This image shows an agarose gel comparing the products of a restriction enzyme single digestion of the IRT2-pHYG and the BCL-XL-pHYG vector. The first lane contains the empty pHYG vector at ~5700BP, lanes 2 and 4 contain the linearized IRT2-pHYG ~7000 BP vector, and lanes 3 and 5 contain the uncut supercoiled IRT2-pHYG vector. Lanes 6 and 8 contain the linearized BCL-XL-pHYG vector ~6200B P, and lanes 7 and 9 contain the uncut supercoiled BCL-XL -pHYG vector. This image confirms our ligation with both genes of interest onto our empty pHYG vector was successful.

Plasmid Map of BCL-XL-pHYG Vector

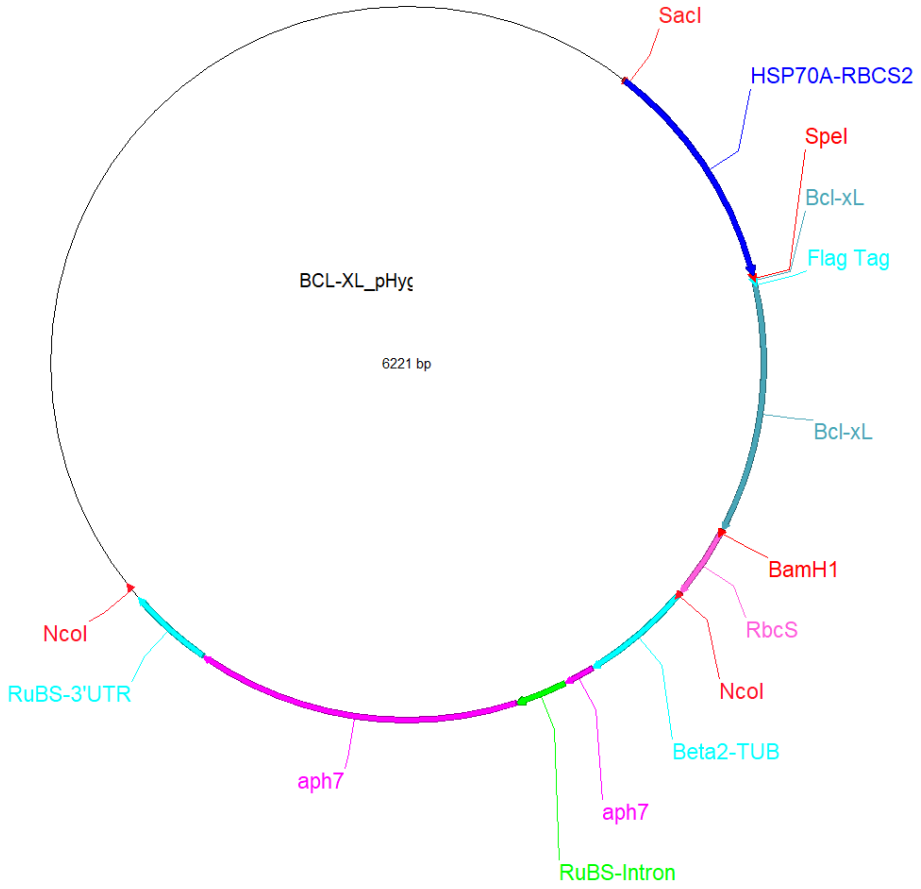

Figure 16. This is a schematic representation of the algal transformation vector used in this study to express the BCL-XL gene. Within the algal replicon it contains all the elements for protein expression in *C. reinhardtii.* Notice that gene is tagged with a flag epitope tag for downstream purification; the gene is inserted between restriction sites BamH1 and Spe1.

Plasmid Map of IRT2-pHYG Vector

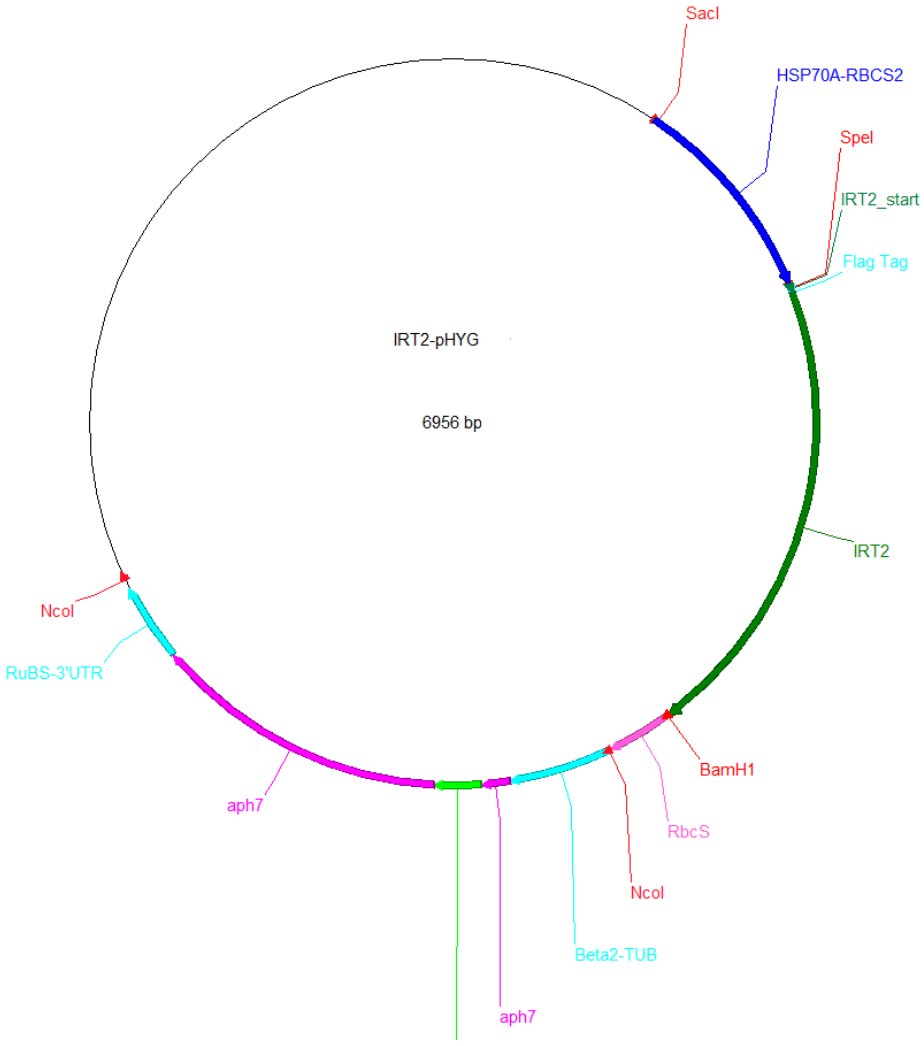

Figure 17. This is a schematic representation of the algal transformation vector used in this study to express the IRT2 gene. Within the algal replicon it contains all the elements for protein expression in *C. reinhardtii.* Notice that gene is tagged with a flag epitope tag for downstream purification; the gene is inserted between restriction sites BamH1 and Spe1.

Chapter 5: Transformation and Subsequent Selection of *Chlamydomonas reinhardtii*.

Once our vector constructs were built and curated appropriately we decided to continue with our algal transformation. In order to do this we had to cultivate our cells to appropriate physiological conditions and follow a very strict transformation protocol.

5.1 Cultivation Conditions

First we grew *C. reinhardtii* CC503 in a T-75 shake flask containing 40 mL of TAP growth media until it reached mid exponential phase. Cell growth was monitored by counting via hemocytometer once at least every 24 hours. Once seed flask had reached mid exponential phase we inoculated a 500 mL photobioreactor containing TAP media with a density of 1×10^5 Cells/mL and monitored growth until the cell density reached $2 - 3 \times 10^6$ Cells/mL. Once cultures reached their desired density they were harvested for downstream applications. All cultures were grown photoautotrophically at room temperature under constant lighting on a revolving platform.

5.2 DNA Treatment Prior to Transformation

We observed earlier that circular plasmids have the tendency to form supercoiled forms of DNA; a biophysical trait which can potentially hinder membrane penetration. In order to circumvent this phenomenon and improve our transformation efficiency we intend to use linearized plasmids while carrying out transformation. In order to linearize our construct we decided to use a blunt cutting enzyme that cut far away from our algal expression cassette; our vector so happened to have one unique Xmn1 site along the *E.Coli* expression cassette and this restriction site was not found in either of our genes of interest; thus we digested both plasmids using this enzyme. In figure 18 we can see where the Xmn1 site is located relative to the algal expression cassette (located in the same place for both constructs); digestion with this enzyme leaves a blunt ended linear fragment with the algal expression cassette intact.

Plasmid Map Showing Blunt Restriction Site for Linearization

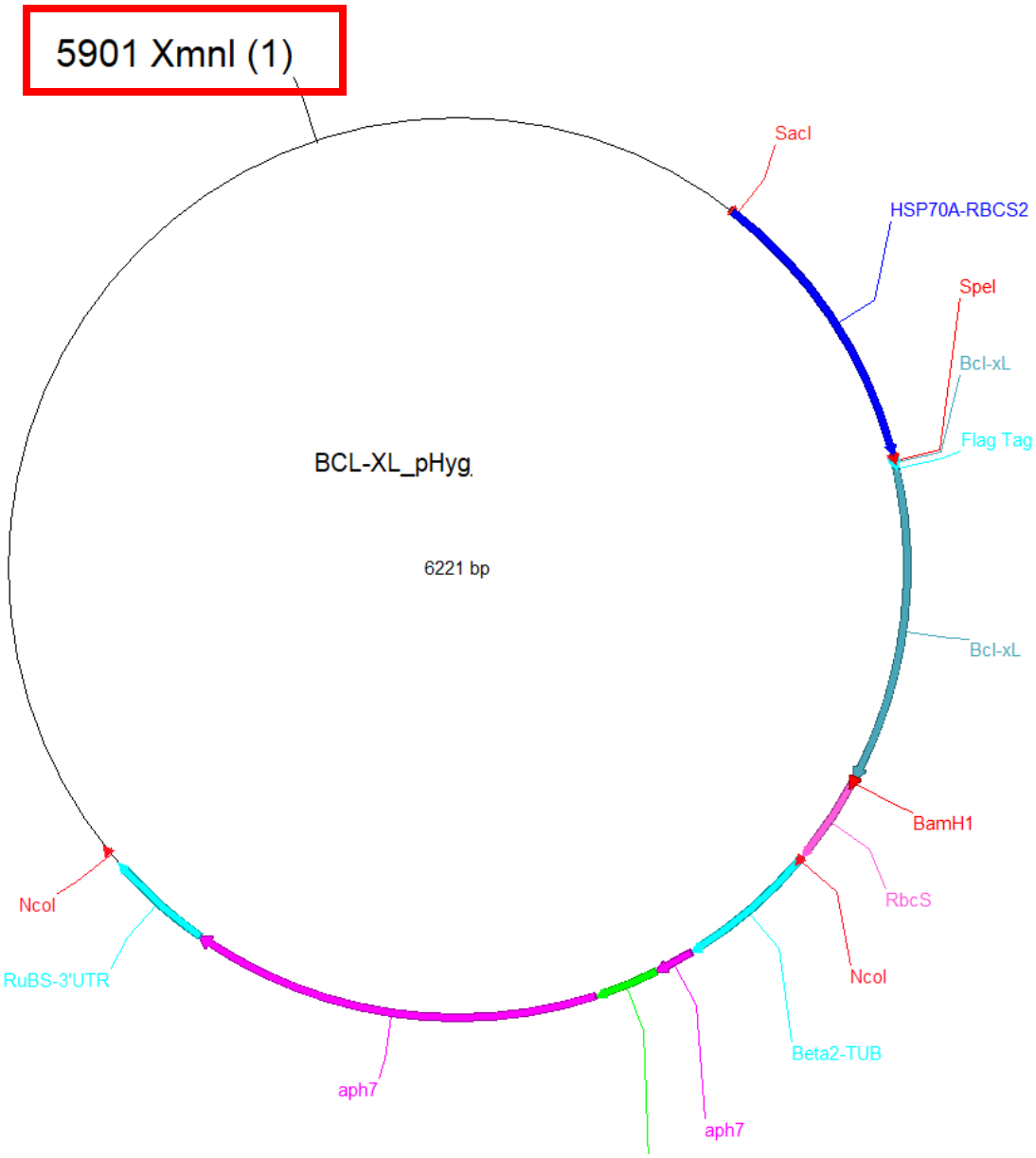

Figure 18. This is a schematic representation of the algal transformation vector used in this study to express the BCL-XL (and IRT2) gene. Notice the Xmn1 Restriction site (red box) along the *E.coli* replicon; this site was chosen because it is sufficiently up and downstream from the algal replicon and treatment would yield a linearized plasmid ready for transformation.

5.3 Electroporation Protocol

Once all prerequisites for transformation have been met (treatment of transforming DNA and culture density) then the process can be carried out. It is very important to note that the transformation process should be done quickly in order to maximize transformation efficiency. The first step in the transformation protocol is to determine cell concentration; from there we estimate the amount of culture volume necessary to have approximately 4 x 10^8 cells in the pellet. Once the culture volume necessary has been determined then we chill cells on ice for 10 minutes. After ice incubation, we centrifuge cells at 2000 x g for 5 minutes at 4 C. Once the cells are in the pellet discard supernatant and resuspend gently on ice in 1 mL of TAP media supplemented with 60 mM sucrose. Once cells are well mixed transfer to a 1.5 mL eppendorf tube. Aliquot 250 µL of suspended cells into new eppendorf tubes on ice and add 1 µg of linearized transforming DNA into each cell sample. Remove samples from ice and incubate in a 16 C water bath for 5 minutes, once incubation has concluded transfer each sample into an individual 4mm gap Electroporation cuvette. Place cuvette in Electroporation scaffold assuring that the electrodes are in contact with the metal sides of the cuvette. Electroporate sample with one pulse at 750 volts, 25 µF, and no resistance (Alternatively: 650 volts, 25 µF, and no resistance).

Incubate sample at room temperature for 10 minutes then take all of sample and transfer to a 15 mL conical tube with 10 mL TAP + sucrose. Lastly, incubate in low light conditions without shaking for 24 hours.

After overnight incubation harvest cells by centrifugation at 2000 g for 1 minute at room temperature; once cells are in the pellet discard supernatant and resuspend the pellet with 1 mL of TAP media. Plate 500 µL of cells on TAP plates supplemented with 15 µg/mL Hygromycin B; ensure that cells are cover the surface area of the plate evenly. Wrap plates in parafilm, invert, and incubate them in reduced light conditions for 3 – 4 days. After 3 – 4 days incubate plates in standard light conditions, colonies should be visible 7 days after initial plate inoculation. Note: it is crucial to work fast, specifically less than an hour should pass between initial centrifugation (harvesting) and electroporation.

5.4 Electroporation Results: Strain CC503

Electroporation with strain CC503 was carried out as directed in the above protocol. In this experiment we used both the 750 and 650 volt regime, and both gave results. The exact electroporation conditions can be observed in table 6. In general, it is good to have a time relatively low time

constant ~ under 10 ms; however, this will vary depending on the ionic strength and sucrose concentration of your electroporation buffer (media).

Table 6: Electroporation Conditions for *C. reinhardtii* Transformation

Strain	Voltage (kV)	Capacitance (μF)	Time Constant (ms)
CC503-BCL-XL-pHYG	.77	25	6.02
CC503-BCL-XL-pHYG	.61	25	12.3
CC503- IRT2-pHYG	.77	25	5.86
CC503-IRT2-pHYG	.61	25	12.6

After electroporation strains were streaked on TAP plates with an appropriate amount of antibiotic; after 7 days of constant illumination many individual colonies began to appear. Once we saw these colonies the subsequent step was to determine which of these contained had incorporated foreign genes at the DNA level. To do so we employed PCR as a diagnostic tool.

5.4.1 Screening transformants via Colony PCR

As mentioned before algal transformation is difficult due to random incorporation of transgenes into the genome. Additionally, epigenetic and cellular mechanics such as RNA interference play a nontrivial role in foreign

gene silencing. Although all of transformants were growing under Hygromycin pressure (wild type strain under same pressure observed no growth) we wanted to choose the transformant with the highest incidence of foreign DNA integration. Thus we employed a method of colony PCR in order to determine our most viable transformant.

In general, we took a small sample from each individual colony and resuspended it 10 μL of a 6% chellex solution. Each sample was then boiled at 95 C for 10 minutes and then centrifuged at max speed in order to pellet cell debris. Samples were removed from centrifuge and placed directly on ice; 2 μL of each sample was aliquoted into each PCR reaction. For this PCR reaction we used a Taq based polymerase and primers appropriate for each target gene. It should be noted that in since IRT2 is a native gene in *C. reinhardtii* we wanted to make sure to target the promoter in our cascade of genes, thus we ran the reverse primer with a primer complementary to the HSP70A-RBCS2 promoter approximately 170 BP upstream of the start IRT2 in the expression cassette. The primer sequence was CTCAACATCTTAAAATGGCCAGGTG and the expected gene fragment was about 1640BP (primers used for BCL-XL sequence specific as it is not a native sequence). Reactions were run according to the programs outlined by

NEB Taq PCR protocols. The annealing temperatures used for each reaction can be appreciated in table 7.

Table 7: Annealing Temperatures used for Taq based Colony PCR

Strain	Annealing Temperature (C)
CC503-BCL-XL-pHYG	57
CC503-IRT2-pHYG	53

After reaction completion samples were analyzed on a 1% agarose gel stained with ethidium bromide. Results from colony PCR can be appreciated in figures 19 and 20, where figure 19 displays results from IRT2 transformants and figure 20 of BCL-XL. In general, lane 1 of both gels is the amplification of each respective control plasmid while the rest of the lanes are results of colony PCR amplification. As expected we see many transformants show positive for IRT2 integration at about 1640 BP and BCL-XL at ~ 740 BP. Notice how many lanes present very faint bands, this is evidence of inefficient DNA integration into the genome and gene silencing events which are known to take place in algae.

Agarose Gel Result of Colony PCR with IRT2

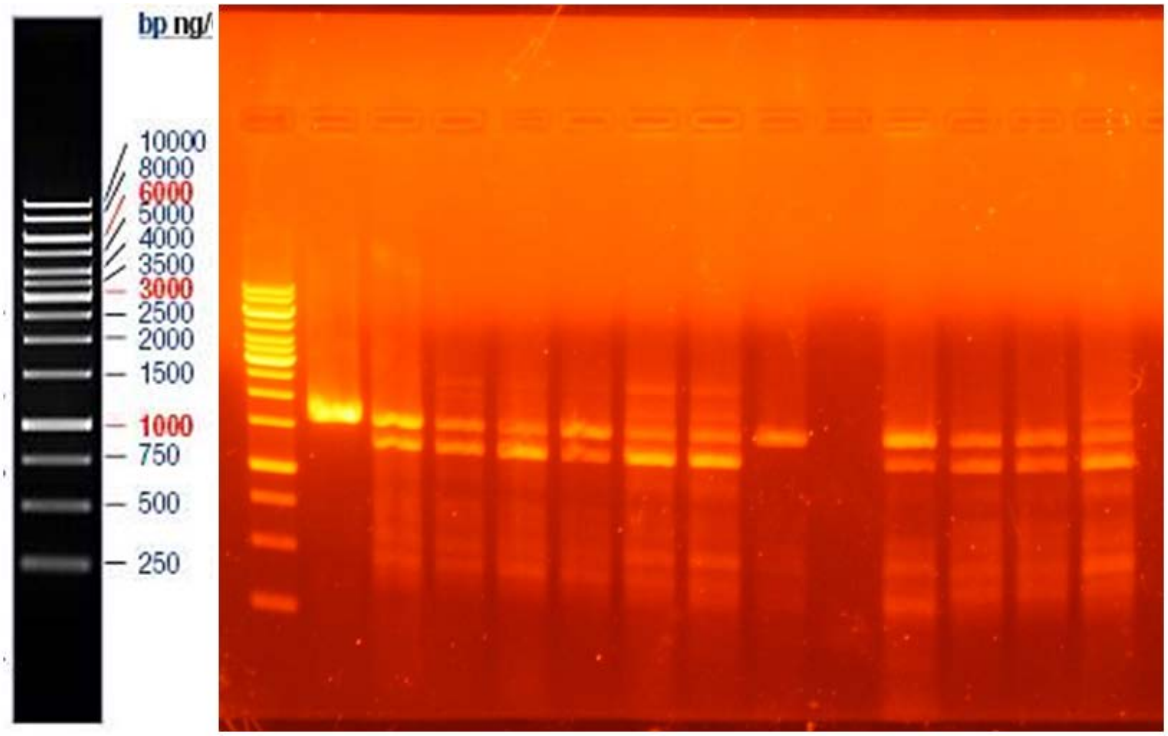

Figure 19. This image shows an agarose gel with the results following colony PCR with algal colonies that passed Hygromycin B selection following transformation with IRT2-pHYG. Lane 1 is an amplification of using the IRT2-pHYG plasmid as a control; it yields a band at ~1640 BP. The rest of the lanes are results of amplification with individual colonies. Lanes 8 and 10 were chosen for further propagation.

Agarose Gel Result of Colony PCR with BCL-XL

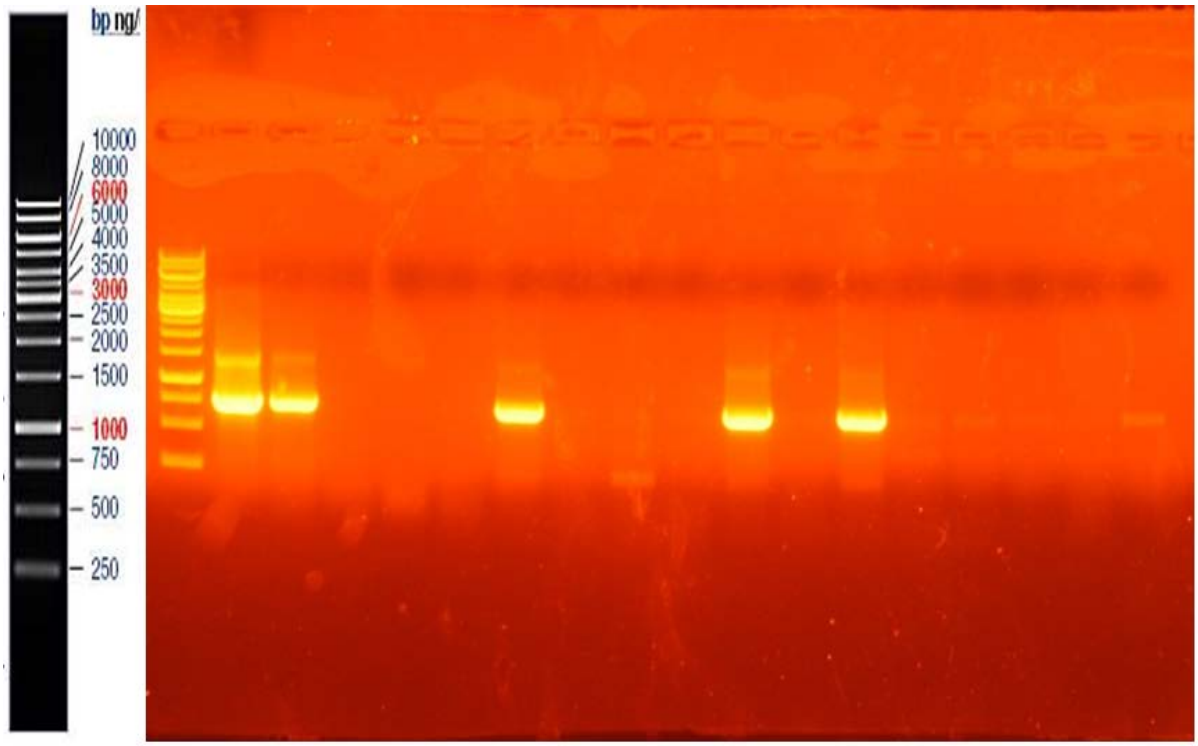

Figure 20. This image shows an agarose gel with the results following colony PCR with algal colonies that passed Hygromycin B selection following transformation with BCL-XL-pHYG. Lane 1 is an amplification of using the BCL-XL -pHYG plasmid as a control; it yields a band at ~740 BP. The rest of the lanes are results of amplification with individual colonies. Lanes 2, 6, 10 and 12 were chosen for further propagation.

5.5 Transformant Subculturing Conditions

After colony PCR results were conclusive we decided to subculture the individual colonies that produced positive results. For IRT2 transformants we chose the colonies corresponding to lanes 8 and 10, for BCL-XL transformants we chose the colonies corresponding to lanes 6 and 10. All of these colonies were subcultured in T-25 culture flasks containing 5 mL TAP media supplemented with 10 μg/mL of Hygromycin B followed by transfer to flasks containing 15 μg/mL of Hygromycin B; we did this in order to increase the stringency of our selection and ensure that only cells expressing our foreign gene cassette at maximal levels could propagate into fully developed cell lines. All cultures were grown photoautotrophically under constant illumination at room temperature on a rotating platform.

Chapter 6: Improving Stress Tolerance in *Chlamydomonas reinhardtii*

As mentioned earlier the goal of this experiment was to improve the physiological qualities of algae in an effort to increase industrial productivity. In the interest of industrial productivity we decided to elucidate a way to improve stress tolerance in algae. As described earlier, industrial cultivation processes are not ideal and encounter many environmental, physical, chemical, and even biological disturbances. Specifically, many times industrial algal cultures find limitations of necessary nutrients such as carbons dioxide, nitrates, light, and other carbons sources. Furthermore, other disturbances such as salt concentration, light intensity, temperature shifts, and contamination are also common in industrial settings. These disturbances cause stress conditions for biological systems, like algae, which can have devastating consequences spanning from reduced cellular productivity to irreversible mutations and even cell death. Therefore, it is clear why engineering a strain with higher stress tolerance is beneficial for industrial cultivation.

Stress affects cells by mediating their metabolism; in cases of extreme stress, apoptotic mechanisms are activated in an effort to keep a healthy cell

population. In order to mitigate the effects of apoptosis we chose to find an inhibition pathway to avoid cell death thus improving overall productivity. We expressed mammalian anti-apoptotic proteins such as BCL-XL in an effort to achieve our goal.

6.1 Apoptosis Inducing Agents and their Effects

In this section we will discuss a couple of factors that are known to induce cell death by various mechanisms. Our goal for this project is to expose our cells to a robust amount of apoptotic triggers in order to verify if 1) our transgenic cell line has better stress tolerance characteristics over the wild type cell line and 2) to determine whether our cell is expressing our protein of interest. We will do this by observing physiological characteristics during growth and also by observing the cellular response to these triggers.

6.1.1 Rose Bengal

Rose Bengal is a stain that is used widely in the medical diagnostic industry in order to detect damaged corneal cells [149]. For our purposes however it will be used as a photosensitizing dye. Rose Bengal works by creating reactive oxygen species that cause photooxidative stress [148].

These oxygen radicals cause membrane and DNA damage which induces apoptosis.

6.1.2 Exposure to Hydrogen Peroxide

Hydrogen peroxide is a strong liquid oxidizing and bleaching agent; in solution it forms free radicals which cause damage to cells. Pointedly, it generates reactive oxygen species such as oxygen, hydroxide radicals and superoxide ions [150]. These radicals cause damage to DNA and membranes such as the mitochondria and the chloroplasts which inhibit photosynthetic efficiency and can trigger the release of cytochrome-C which triggers apoptosis.

6.1.3 Sudden Salt Exposure

Algae are a diverse species that span fresh water and salt water environments. Freshwater algae, when exposed to salt water, limit their growth; this happens because salt exposure induces osmotic, ionic, and oxidative stress [151]. Osmotic pressure affects cells by determining how much water is in them; specifically in hypotonic solutions cells swell, and in hypertonic ones they shrivel up relative to an isotonic solution. Moreover, addition of salt into media causes an increase in ionic strength; that is, the concentration of Na and Cl ions in the media increases causing the cell to go

under stress. Lastly, oxidative stress causes irreversible damage to all cellular components; these reasons combined cause stress in cells which trigger apoptosis [152].

6.1.4 Exposure to Radiation.

Exposure to radiation by organic tissues leads to adverse effects such as tissue necrosis and cellular damage. Radiation comes from various sources; one is the decomposition of unstable elements which emit alpha, beta, and gamma particles. Other forms of radiation are non-ionizing such as UV light; moreover, many forms of radiation are harmful to tissues and cause extensive cellular damage.

UV radiation is the most prominent carcinogen and form of cellular damage in our native environment [153]. When cells or tissues are exposed to high doses of UV radiation adverse effects such as oxidative stress, protein denaturation, and irreversible DNA damage occur [153]. While prolonged exposure to any form of UV radiation has adverse implications; short wave UV-C radiation is by far the most damaging and quick acting. Specifically, UV-C radiation causes irreversible DNA damage to cells by causing pyrimidine dimers and other detrimental photoproducts that inhibit

or sabotage DNA and RNA replication machinery [154]. On top of inhibiting proper protein synthesis, UV-C radiation causes an array of harmful mutations that trigger apoptosis therefore this appealed as an experimental potential. In this study we will be focusing on the adverse effects of UV-C radiation.

6.2 Comparative Response to Environmental Stress between Transgenic and Wild Type *Chlamydomonas reinhardtii*

In the previous section we discussed many methods of inducing apoptosis in tissues. In this section we will analyze the response of different lines of *C. reinhardtii* to these stresses hoping that our transgenic strain copes more effectively with environmental stress. Along with stress conditions a comparative growth under normal conditions will also be included. In general all cultures were grown in T-25 culture flasks containing 5 mL of TAP media under constant lighting in photoautotrophic conditions on a rotating platform.

6.2.1 Comparative Growth under Normal Culture Conditions

In this experiment we sought to compare and determine differences between the physiological growth behaviors of wild type and genetically

modified CC503. To do this we inoculated shake flasks with exponentially growing cells and allowed them to grow over a span of 18 days. We observed that the wild type strain exhibited is marginally quicker lag phase and grew faster than the transgenic strain through exponential phase. The genetically modified strain grew appreciably slower through exponential phase which is to be expected since production of apoptotic proteins is an energy intensive process which shocks the algal metabolism and causes it to behave differently; in this case it slows the growth rate. However, once growth levels off with the wild type strain we can still observe the transgenic strain exhibiting sustained growth. This causes the transgenic strain to grow to an overall higher cell density before it enters decline phase. It should also be noted that the wild type strain enters decline phase much more rapidly. This behavior can be appreciated in figure 21; data is representative of 4 independent experiments with error bars representing the standard error of the mean. Representative data can be found in appendix A.

Growth Comparison in Long Term Culture

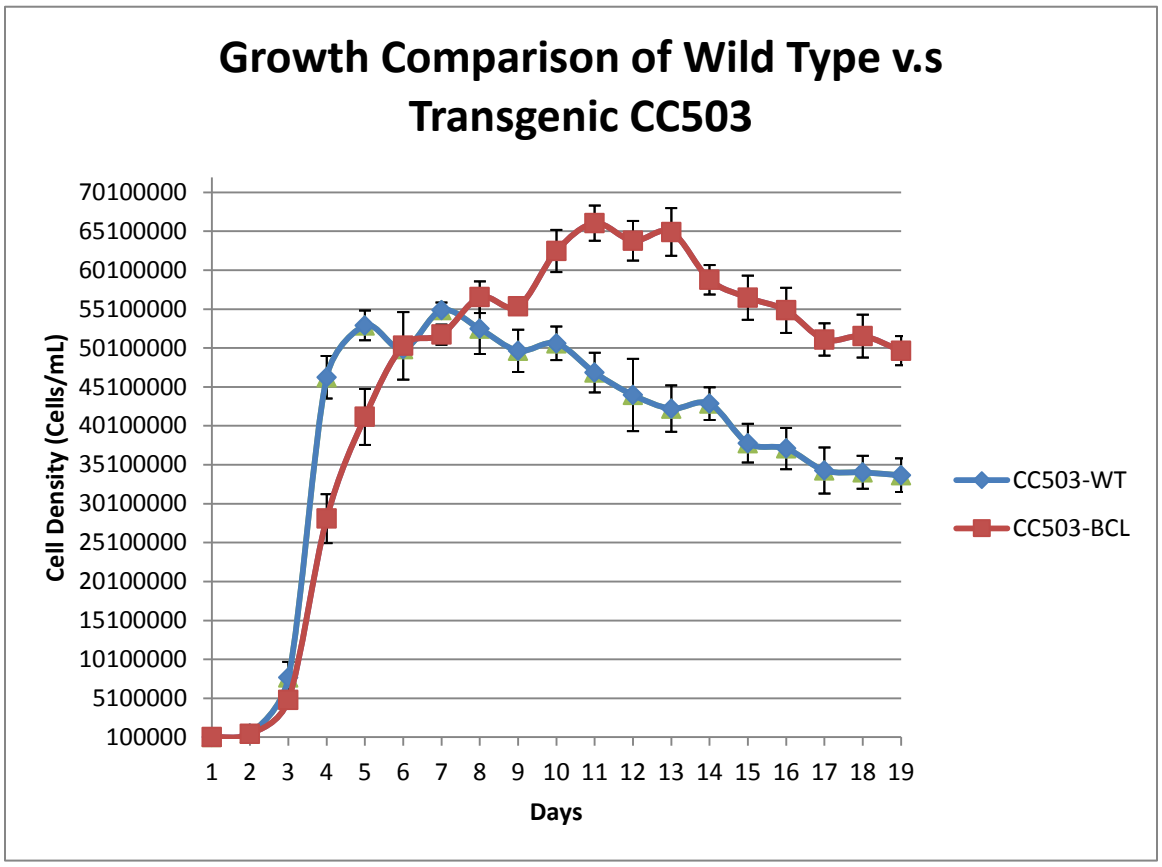

Figure 21. In this figure we can appreciate the growth characteristics of both transgenic and wild type *C. reinhardtii* CC503. Exponentially growing cells were inoculated and growth was observed over a period of 19 days. Error bars represent standard error of the mean, data representative of 4 independent biological replicates.

6.2.2 Response to Rose Bengal Exposure

Cells were inoculated at a density of 1 x 10^7 cells/mL and allowed to grow until mid exponential phase. At this point Rose Bengal (RB) was added into the culture flask and cellular growth was observed over the next few days. Specifically, we made a 200 μM Rose Bengal in Isopropanol stock solution and aliquoted the appropriate volume to have a final 2 μM RB concentration in the culture media. RB was added on the third day, results comparing the growth of wild type vs. transgenic cells can be appreciated in the shaded region of figure 22. Notice how the wild type cell density decreases in response to Rose Bengal addition and how it is suppressed until the RB is degraded by light. Also notice how although growth is hindered, there is no significant reduction in the transgenic population. Results are representative of 2 biological replicates with error bars showing the standard error of the mean, for individual experiments see appendix A.

Comparative Growth of BCL-XL Transformants to Rose Bengal Exposure

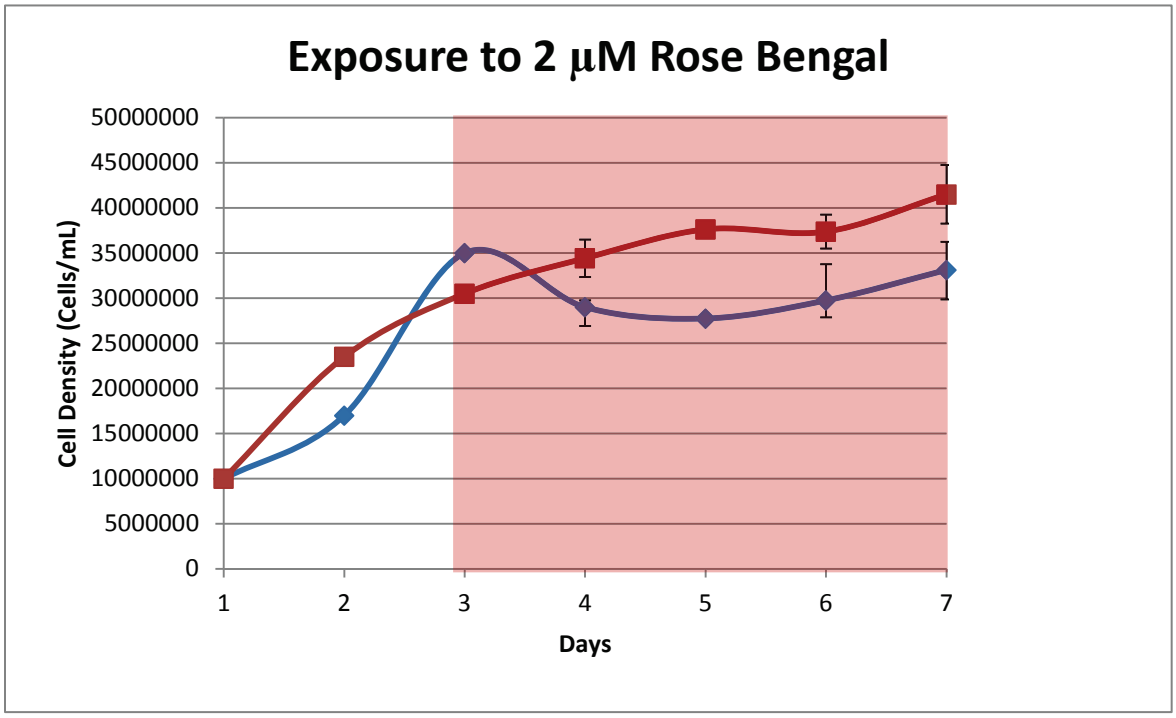

Figure 22. In this figure we can appreciate the effect that photooxidative stress induced by Rose Bengal has on the populations of both transgenic and wild type *C. reinhardtii* CC503 (Pink shaded region). Stress was induced by 2 µM addition of Rose Bengal on the third day of culture and followed by growth analysis. Error bars represent standard error of the mean, data representative of 2 independent biological replicates.

6.2.3 Response to H_2O_2 Exposure

Cells were inoculated at a density of 1 x 10^7 cells/mL and allowed to grow until mid exponential phase. At this point Hydrogen peroxide was added into the culture flask and cellular growth was observed over the next few days. Specifically, we used a 3% stabilized hydrogen peroxide stock solution and aliquoted the appropriate volume to have a final 1 mM H_2O_2 concentration in the culture media. H_2O_2 was added on the third day, results comparing the growth of wild type vs. transgenic cells can be appreciated in the shaded region of figure 23. Notice how the wild type cell density drops in the presence of H_2O_2; also notice how the transgenic cell line experiences retarded growth but no population decline. Lastly, notice how on the final days the cell density rises again, this is because the concentration of H_2O_2 in the media has dropped due to decomposition, allowing cellular growth to resume. Results are representative of 2 biological replicates with error bars showing the standard deviation from the mean, for individual experiments see appendix A.

Comparative Growth of BCL-XL Transformants to H_2O_2 Exposure

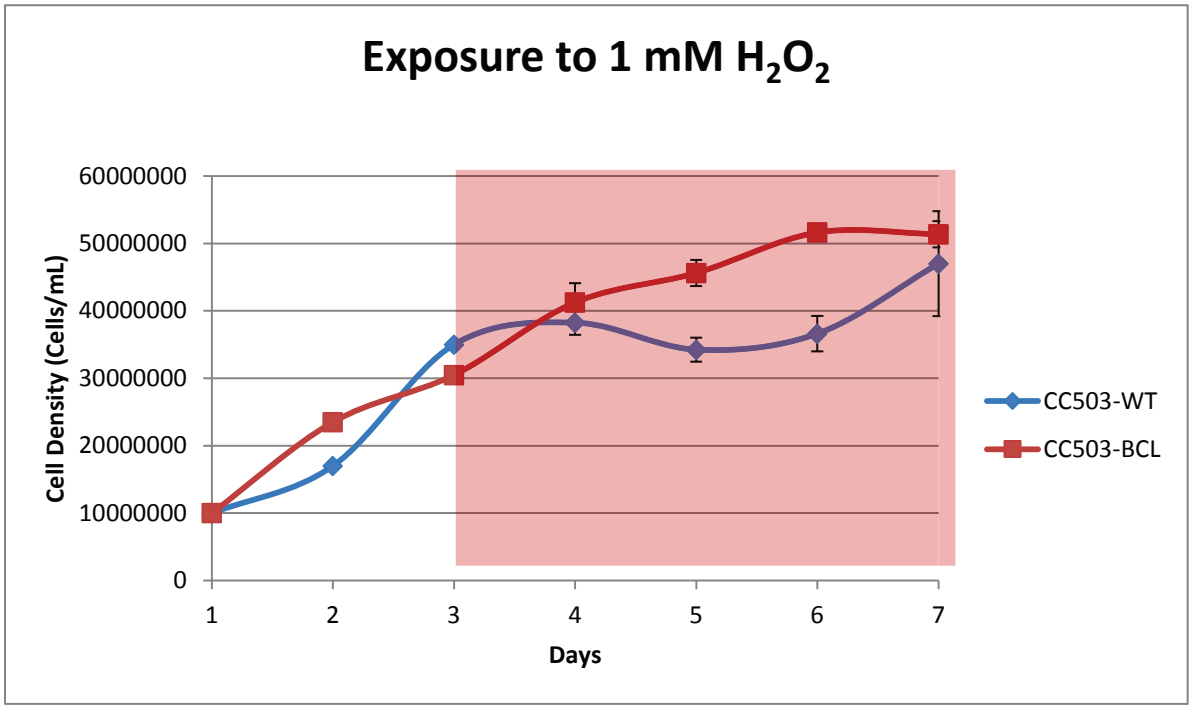

Figure 23. In this figure we can appreciate the effect that photooxidative stress induced by Hydrogen Peroxide has on the populations of both transgenic and wild type *C. reinhardtii* CC503 (Pink shaded region). Stress was induced by 1 mM addition of H_2O_2 on the third day of culture and followed by growth analysis. Error bars represent standard deviation from mean, data representative of 2 independent biological replicates.

6.2.4 Response to NaCl Exposure

Cells were inoculated at a density of 1 x 10^7 cells/mL and allowed to grow until mid exponential phase. At this point NaCl was added into the culture flask and cellular growth was observed over the next few days. Specifically, we made a 1 M NaCl stock solution and aliquoted the appropriate volume to have a final 100 mM NaCl concentration in the culture media. The salt water was added on the third day, results comparing the growth of wild type vs. transgenic cells can be appreciated in figure 24. In this experimental regime we sought to shock cells by causing a sudden change in osmotic pressure; as seen in the results both cell lines experienced a sharp decline in population following salt addition. Interestingly, the transgenic population was more resistant to death and adapted to the new conditions more rapidly than the wild type population. Results are representative of 2 biological replicates with error bars showing the standard deviation from the mean, for individual experiments see appendix A.

Comparative Growth of BCL-XL Transformants to Osmotic Pressure Shift

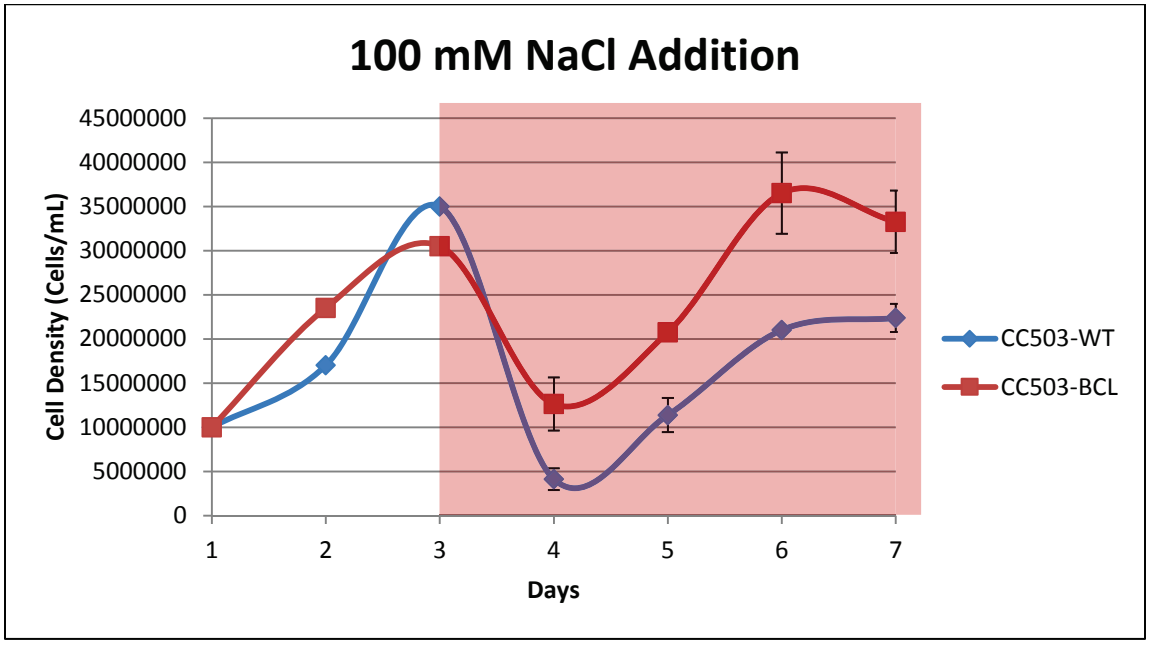

Figure 24. In this figure we can appreciate the effect that stress induced by a sudden change in osmotic pressure has on the populations of both transgenic and wild type *C. reinhardtii* CC503 (Pink shaded region). Stress was induced by 100 mM addition of NaCl on the third day of culture and followed by growth analysis. Error bars represent standard deviation from the mean, data representative of 2 independent biological replicates.

6.2.5 Response to UV-C Exposure

Cells were inoculated at a density of 1 x 10^7 cells/mL and allowed to grow until mid exponential phase. At this point cells were removed from their respective flask and transferred to a sterile 6 well plate. The plate was placed within a biological safety cabined fitted with a bulb capable of producing short wave UV-C radiation and exposed for 10 minutes. After exposure cells were returned to their respective flasks and cellular growth was observed over the next few days. Exposure to UV-C was conducted on the third day, results comparing the growth of wild type vs. transgenic cells can be appreciated in figure 26. In this experimental regime we sought to shock cells by causing DNA and cellular damage through radiation in an effort to induce apoptosis. The results of this experiment were impressive as the wild type cell line experienced a sharp decline in population and various phonotypical changes following exposure. Interestingly, the transgenic population was more resistant to death and continued to grow following treatment. This experiment was conducted twice and generated reproducible results. Results are representative of 3 biological replicates with error bars showing the standard deviation from the mean, for individual experiments see appendix A. Additionally, figure 25 serves as qualitative evidence documenting the physiological changes the cells undergo after UV treatment.

Physiological Response to UV-C Radiation

Figure 25. These images show the physiological changes that both cell lines undergo after 10 minute treatment with UV-C radiation. In both images the three leftmost cultures are the BCL-XL expressing strains while the three rightmost are CC503 wild type. Notice how after the first day there is evidence of cell death and chlorosis in the wild type population; by the 4th day wild type algae are not viable.

Comparative Growth of BCL-XL Transformants to UV-C Exposure

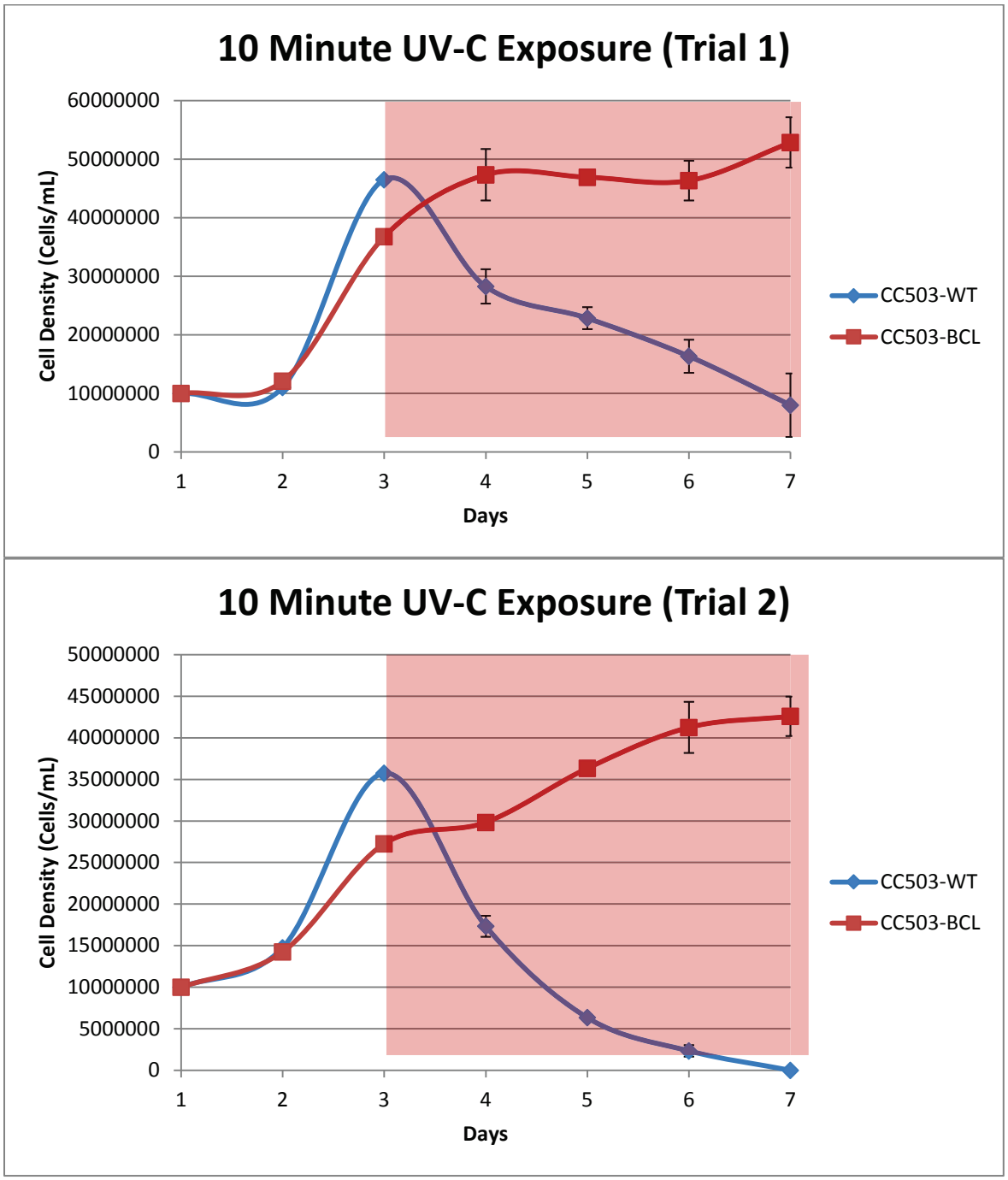

Figure 26. In this figure we can appreciate the effect that stress induced by UV-C radiation has on the populations of both transgenic and wild type *C. reinhardtii* CC503 (Pink shaded region). Stress was induced by 10 minute irradiation on the third day of culture and followed by growth analysis. Error bars represent standard deviation from mean, data representative of 3 independent biological replicates.

6.3 Detecting RNA Level Expression

In order to support life cells must take detailed instructions coded into their genome and produce functional proteins. This transition from DNA to RNA and eventually to proteins is labeled the central dogma of biology and is essential for cell survival and proper function. In the interest of improving industrial productivity we are very interested in introducing transgenes into algal host organisms in an effort to produce non-native recombinant proteins that are functional. In order to confirm expression at the mRNA level we employed the use of RT-PCR. We extracted total mRNA from transgenic *C. reinhardtii* cells and wild type strains as a control. In order to ensure DNA contamination would not cause non-specific amplification we treated the RNA sample with DNase 1, then we subjected the extract to RT-PCR conditions as outlined earlier in this work. The results obtained can be appreciated in figure 27. The bands seen show the amplification of the BCL-XL-pHYG plasmid in lane 1, the appropriate molecular weight for the BCL-XL cDNA ~ 740 BP in lanes 2 and 3. Notice how the wild type strain does not show expression of this discrete band in lane 4.

Comparative RT-PCR Results: CC503-BCL-XL vs. Wild Type

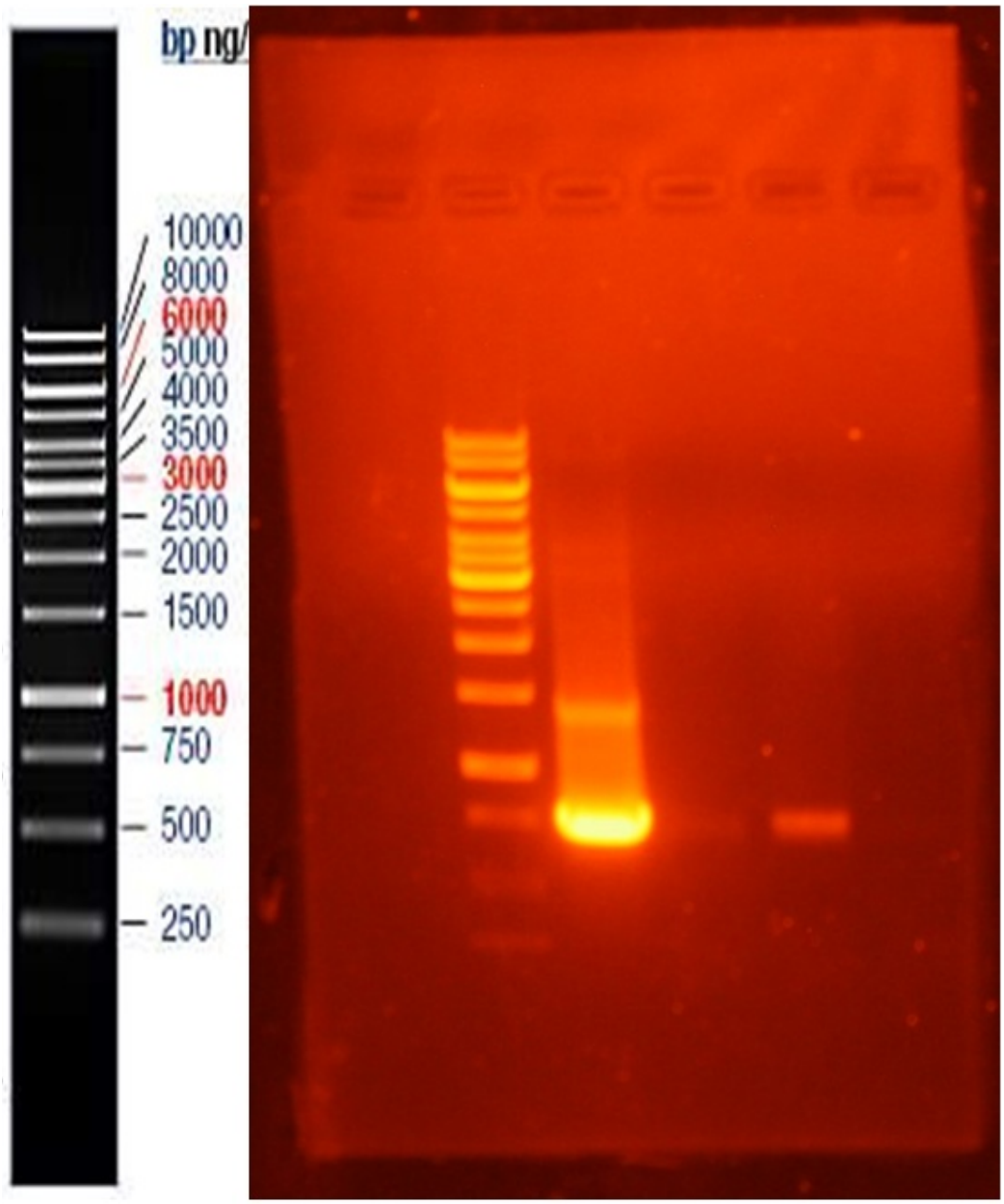

Figure 27. This image shows an agarose gel with the results following RT-PCR comparing algal cultures expressing BCL-XL-pHYG and wild type CC503. Lane 1 is an amplification of using the BCL-XL -pHYG plasmid as a control; it yields a band at ~740; Lanes 2 and 3 show the same band following RT-PCR with transgenic cells and lane 4 shows the result of amplification with CC503-WT.

6.4 Significance of Observed Results

Earlier in this work extensive background was given on the industrial benefits of using algal biomass for production of biofuels and other high value bio-products including recombinant proteins and carotenoids such as astaxanthin. We went over a short history of biofuels, the algal cultivation process and we outlined the shortcomings of industrial algal biomass production. We specifically focused on the fact that industrial algal culture hardly ever meets optimal growth conditions and that the biologics are often put under conditions of stress. Moreover, we discuss the different kinds of stress and what kind of behaviors they induce in organisms, like apoptosis. We also discussed other bottlenecks in production such as the issue of dewatering algae.

We presented two possible regimes that used genetic and metabolic engineering as a basis in order to address these industrial shortcomings. Specifically, we chose to express mammalian anti-apoptotic proteins and to over express membrane coupled iron transporters in algae. After giving background on how each respective motif affected cell physiology and explaining the intended goal a thorough protocol was given explaining the steps involved in producing appropriate expression vectors for algal

transformation. Additionally, an explicit reproducible protocol for the stable transformation of *C. reinhardtii* was included. Lastly, we went over selection methods in order to isolate the transgenic strain and propagate it without wild type contamination.

Once we confirmed via colony PCR that our isolated transgenic cell line contained our transgene at an appreciable level we decided to put it through various experimental regimes that mimicked stresses commonly associated with algal cultivation. Specifically, we sought to create a robust amount of scenarios that induced photooxidative stress which could cause cellular damage leading to apoptosis; we chose to use photosensitizing dyes, adding free radicals, and inducing reactive oxygen species generation via irradiance and sudden osmotic shock. Additionally, through the use of direct UV-C light we sought to damage DNA directly in order to investigate and compare the physiological response to mutations between cell lines; needless to say our transgenic strain outperformed the wild type strain in all scenarios.

Four different stress tests were conducted and one long term culture test was conducted to determine differences in growth characteristics

between the cell lines. All of these tests gave positive results and showed that although the transgenic cell line has an inferior growth rate, its' stress coping capabilities are significantly improved. Specifically, when exposed to reactive oxygen species producing chemicals such as Rose Bengal and Hydrogen Peroxide the transgenic cell line slowed its growth rate but did not experience a decline in population relative to the wild type cell line. This demonstrates that the transgenic cell line is producing the recombinant protein at functional levels to inhibit apoptosis activation by free radicals. Moreover, when both strains are subjected to a sudden change in osmotic pressure it becomes evident that the transgenic cell line is not only more resistant to osmotic stress but it also adapts to the new environmental conditions more rapidly and outgrows the wild type strain. This quality is extremely attractive for industrial and bioremediation applications because it allows these cells to be grown adequately in saltwater and even wastewater. Furthermore, when exposed to UV-C radiation, which causes direct damage to DNA thus inducing apoptosis, the transgenic cell line blows the wild type line out of the water; that is, the transgenic cell line continues to grow while the wild type goes into a stark decline. This is another extremely attractive feature for industrial cultivation because it proves that the transgenic strain has far superior tolerance to more realistic circumstances encountered in

outdoor cultivation such as stress produced by high irradiance. Lastly, through long term growth analysis it becomes evident that although the transgenic cell line initially experiences slower growth, it will grow to a higher cell density and will not enter decline phase as quickly as the wild type strain.

Based on the various stress tolerance experiments performed and the comparative physiological responses between cell lines it is clear that BCL-XL confers a robust tolerance to many facets that induce cell death. Mainly, BCL-XL confers tolerance to induction of apoptosis via DNA damage, mutation accumulation, and cellular damage caused by photooxidative stress and reactive oxygen species. Furthermore, we can confirm stable DNA integration of the transgenes via colony PCR and selective cultivation conditions of the transformants even after countless rounds of cell division, indicating that this is a stable cell line. Moreover, through RT-PCR we are able to detect expression of the transgene at the mRNA level indicating that the algal cellular machinery is localizing to the place of foreign gene integration and transcribing it effectively.

6.5 Conclusions and Future Directions

The goal of this project was to confer more industrially attractive properties to *Chlamydomonas reinhardtii* through genetic and metabolic engineering. Specifically our goal was two-fold; we sought to increase stress tolerance by overexpressing the mammalian anti-apoptotic protein BCL-XL and to increase intracellular iron concentrations by overexpressing IRT2: a membrane coupled iron transporter. As seen in the text 2 vectors for foreign gene expression were successfully built and curated; following transformation and subsequent selection we were able to confirm nuclear transgene integration of both constructs via colony PCR. Once a stable BCL-XL cell line was developed we subjected it to an extensive array of tests which sought to probe its resistance to photooxidative stress and its adaptability to osmotic stress. We found that the transgenic cell lines expressing the anti-apoptotic motif performed better than wild type CC503 when exposed to stresses like Rose Bengal, Hydrogen Peroxide, Salt addition, and UV-C irradiation. We also found that the transgenic cell lines grew slower but to a higher final cell density and fell into decline phase later relative to the wild type strain. Furthermore, through RT-PCR we were able to confirm RNA level BCL-XL expression in the transgenic cell lines.

Although there is an overwhelming amount of physical evidence suggesting that the transgenic cell line is more resistant to stress there are still a few trajectories that would fully confirm this result. For example, a western blot analysis would prove that the cell is expressing the transgene at all biochemical levels (DNA, RNA, and Protein). Furthermore, performing a comparative apoptosis assay after apoptotic insult would prove that the recombinant protein is of a functional form in algae. Following this work, it would be beneficial for the research community to better elucidate PCD pathways in algae in order to fully understand the apoptosis mechanism and further improve the strain. Moreover, overexpressing anti-apoptotic markers is an energy intensive process that shocks and alters the metabolism of mammalian cells; it would be beneficial to investigate the metabolic toll that overexpression of this peptide has on algal cells. Lastly, much work was done on creating a stable IRT2 overexpressing cell line; once this was accomplished these cells were grown on media with differing iron concentrations (50, 100, 150 μM). These samples are currently frozen and awaiting ICP-MS analysis in order to determine whether or not intracellular iron concentrations are different compared to the wild type cells. Following ICP-MS analysis these cells should be probed for magnetic susceptibility to elucidate whether or not magnetophoresis is feasible.

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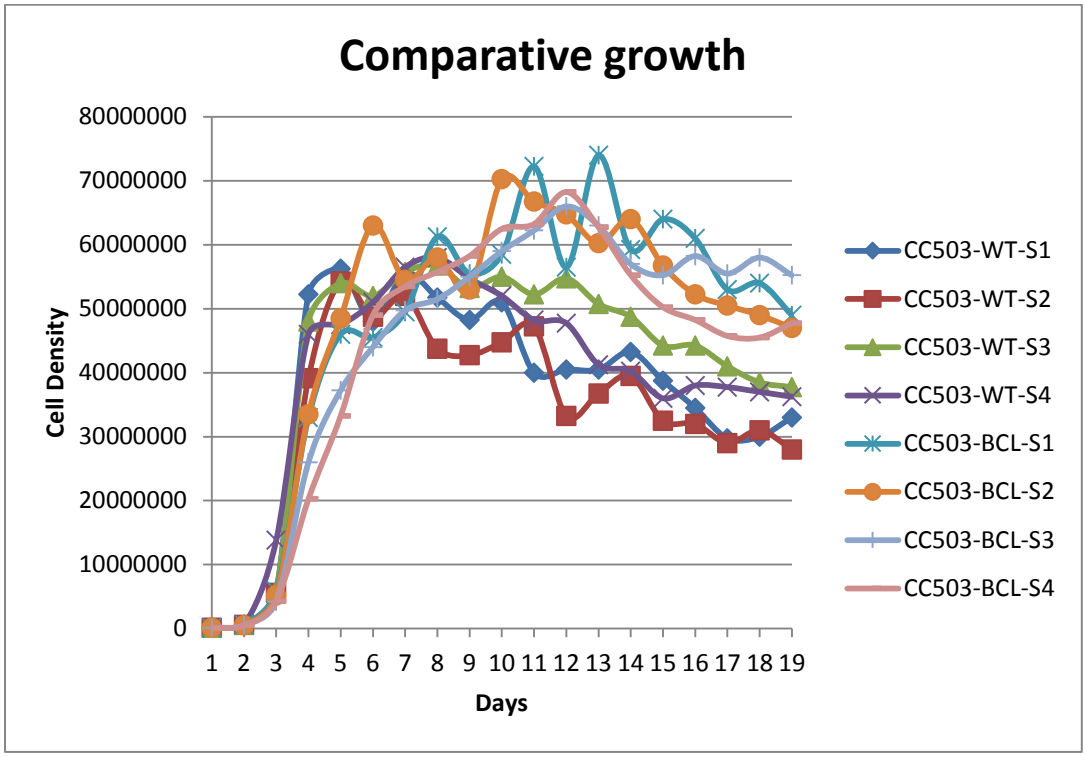
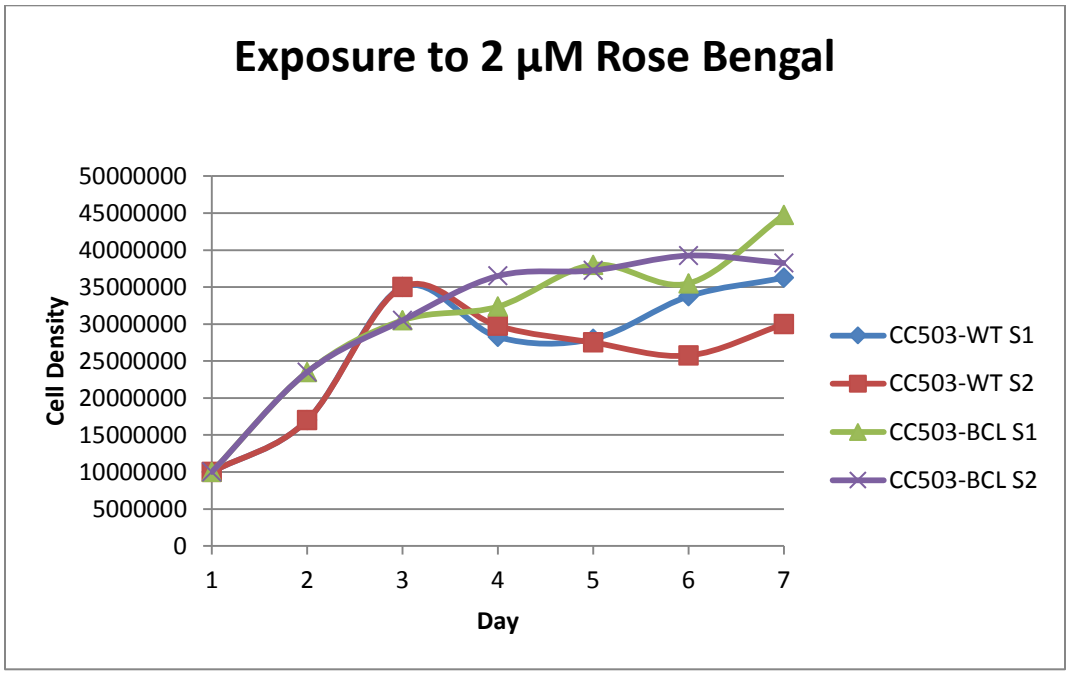
Appendix A: Supplemental Information

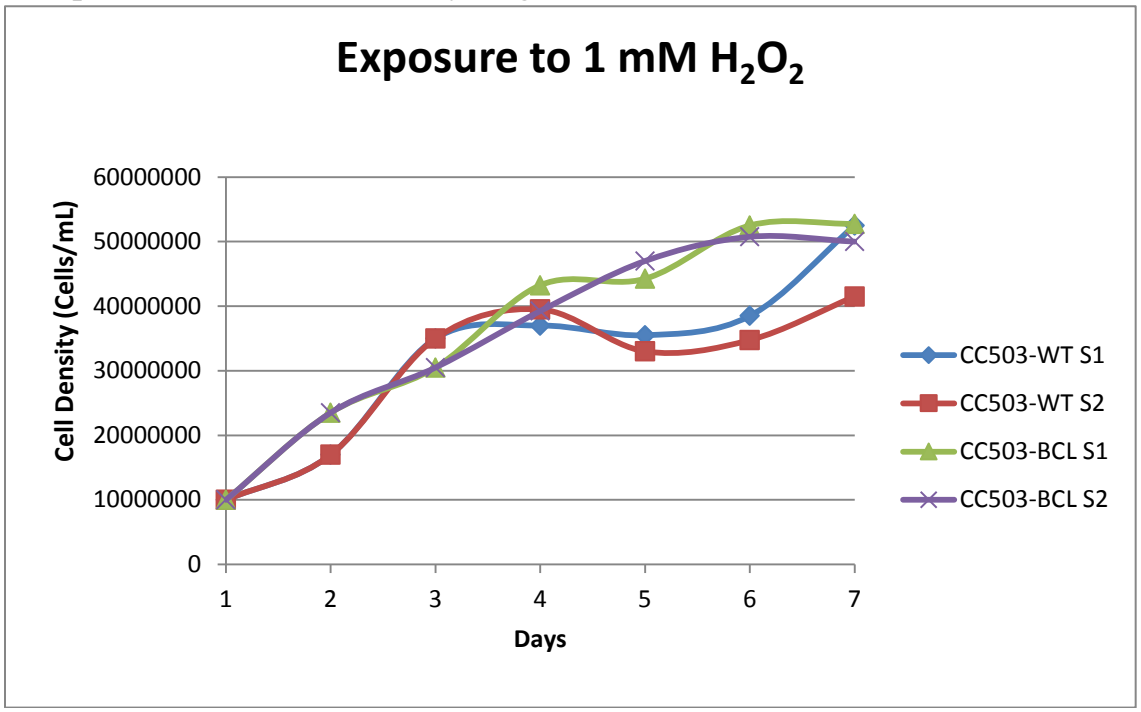
Comparative growth during long term culture: For Growth Data ask for supplemental excel file.



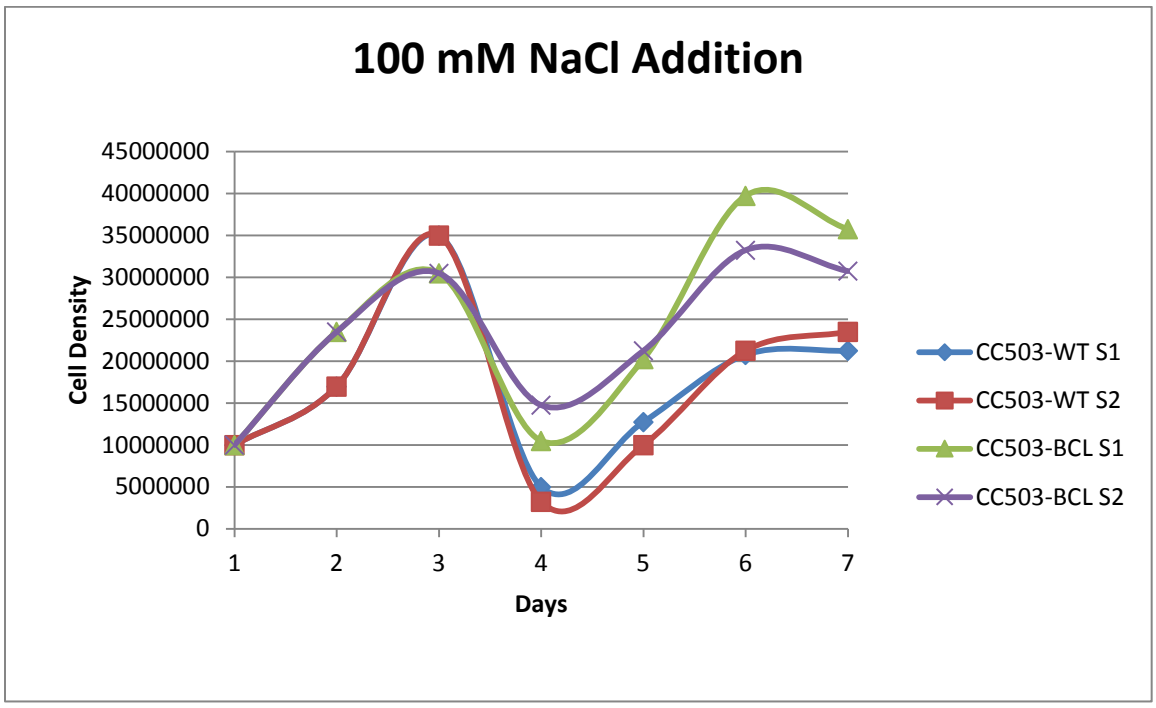
Comparative Growth under Rose Bengal Stress.



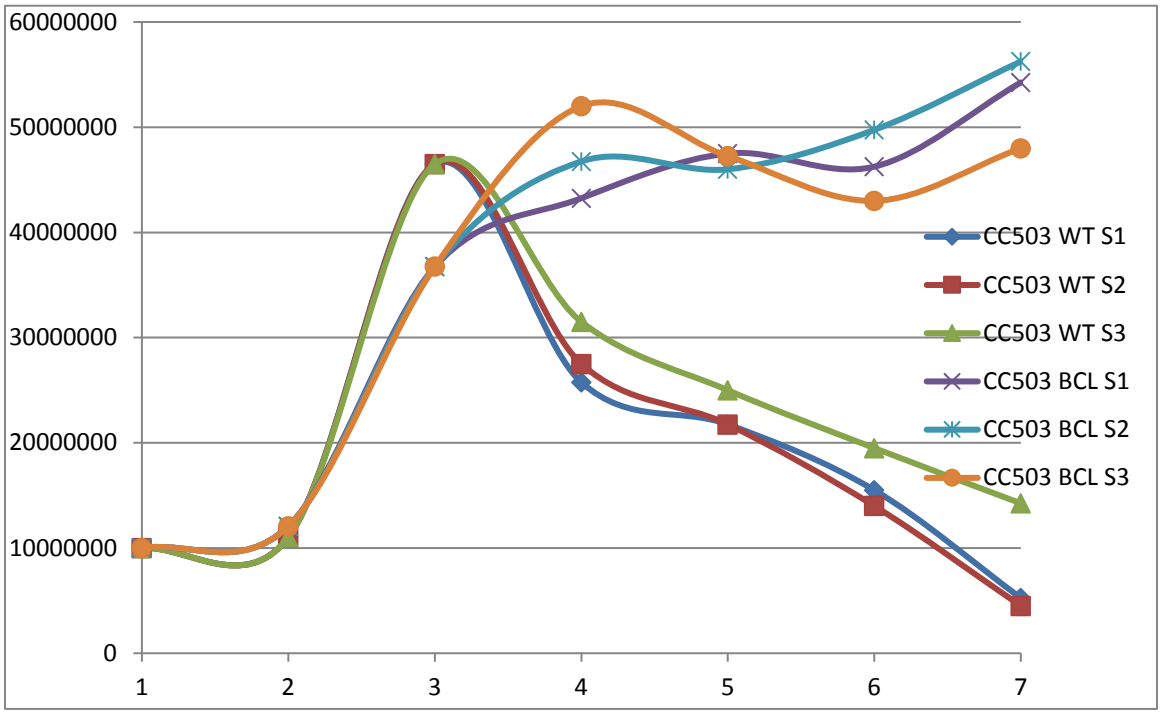
Comparative Growth under Hydrogen Peroxide Stress.

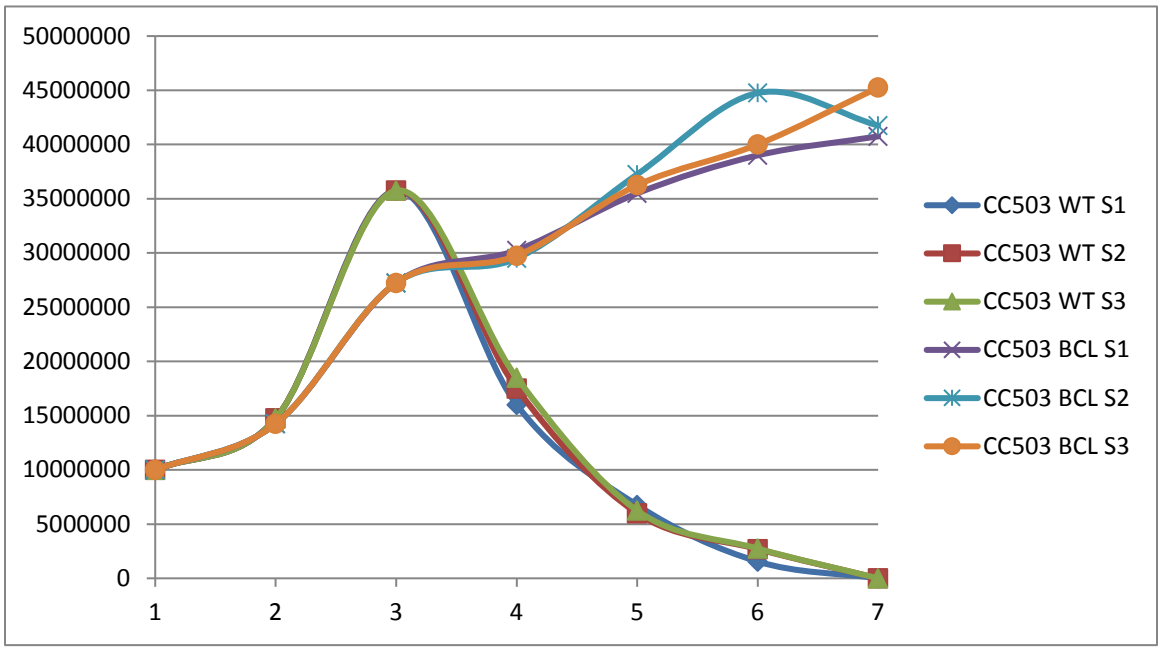


Comparative Growth under Osmotic Stress.



Comparative growth after UV-C Exposure.





VITA

Luis Francisco Garcia Ulloa was born to Marialuisa Garcia Ulloa and Luis Ernesto Garcia Lizarraga on February 15, 1994 in North Hollywood, California. He graduated from Cathedral college preparatory high school in downtown Los Angeles in 2012, after which he moved across the country to attend Johns Hopkins University in Baltimore, Maryland. He studied Chemical and Biomolecular engineering as undergraduate and during his junior year he joined the algae team in the Betenbaugh lab. During his first year of research he worked on a project aiming to establish a phylogenetic standard using 18s-rRNA sequences amongst over 100 species of microalgae and cyanobacteria. During his senior year he worked on identifying algal strains that grew under an excess of iron and he wrote a chapter for the up and coming version of Perry's handbook for chemical engineers on Integrated Cell Culture Product Recovery. He completed his Bachelor's degree in Chemical and Biomolecular Engineering in May 2016, after which he continued onto his masters in the same field at Johns Hopkins University. As part of his masters' research he made a cell line of *Chlamydomonas reinhardtii* with improved stress tolerance by transforming it with the BCL-XL peptide. He completed his masters' in Chemical and Biomolecular engineering in October, 2017. Currently, he is working on publishing a patent.